ISOLATION AND CHARACTERIZATION OF FLUORIDE RESISTANT MAMMALIAN GUT BACTERIA

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

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The entire dissertation work has been done under my supervision and this work has not been submitted elsewhere for a degree.

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DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled "**Isolation and Characterization of Fluoride Resistant Mammalian Gut Bacteria**" in partial fulfilment of the requirements for the award of the degree of Master of Science in Biotechnology is an authentic record of my own work carried out during the period from **January, 2025** to **May, 2025** under the supervision of **Dr. Souryadeep Mukherjee**, Department of Life Sciences, Presidency University.

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ABSTRACT

Fluoride, while beneficial for dental health in low concentrations, poses a significant risk to human health when exposure exceeds recommended limits, particularly through contaminated groundwater. Among the emerging concerns is its potential impact on the gut microbiota, a complex microbial ecosystem essential for digestion, immunity, and overall health. Disruption of this microbial balance, known as dysbiosis, has been linked to various metabolic and inflammatory disorders. Recent studies suggest that chronic fluoride exposure can lead to shifts in gut microbial composition, reducing beneficial bacterial populations and enhancing the growth of potentially pathogenic or fluoride-resistant strains. Additionally, studies in rodents have demonstrated that fluoride exposure reduces microbial diversity and alters the abundance of major bacterial phyla such as Firmicutes and Bacteroidetes, which can affect short-chain fatty acid production and gut barrier integrity. While several investigations have focused on environmental isolates, the isolation and characterization of fluoride-resistant bacteria from the mammalian gut remain limited. Exploring these microbial adaptations could offer insights into microbial resilience, host-microbe interactions under chemical stress, and the possible link between fluoride resistance and antibiotic resistance. Furthermore, understanding the molecular basis of fluoride resistance may contribute to the development of targeted probiotics and inform public health strategies, particularly in regions with endemic fluorosis. This study underscores the significance of studying fluoride-resistant gut bacteria in mammals and calls for more in-depth research into their physiological roles, resistance mechanisms, and broader implications for human health.

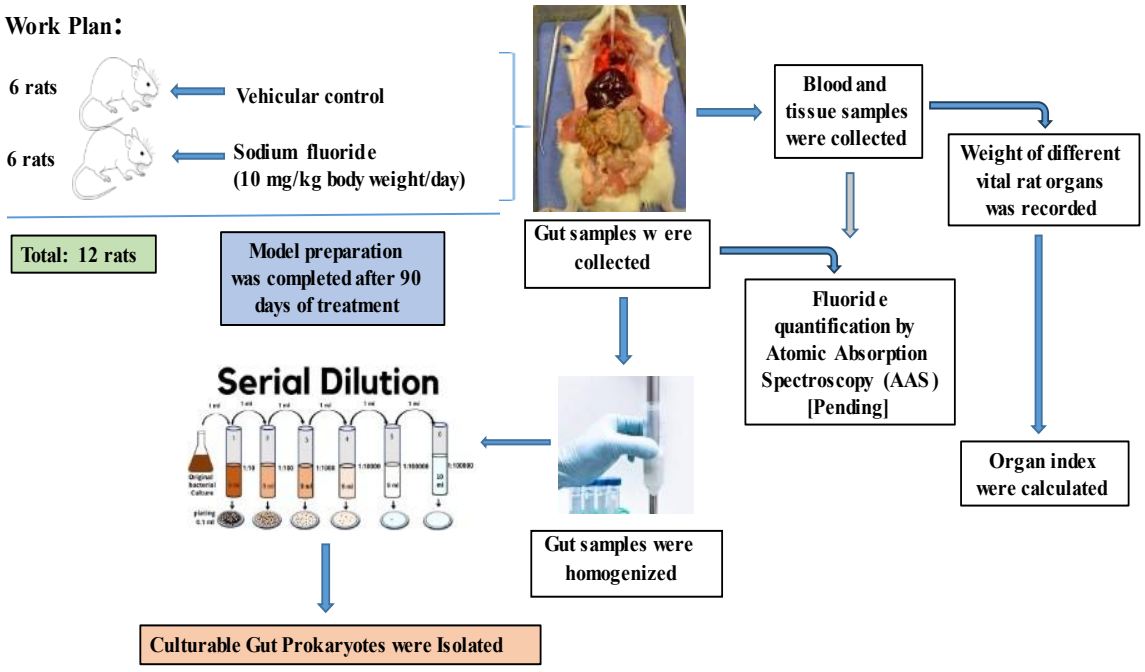

Work Plan:
6 rats
Vehicular control
6 rats
Sodium fluoride
(10 mg/kg body weight/day)
Total: 12 rats
Model preparation was completed after 90 days of treatment
Gut samples w ere collected
Blood and tissue samples were collected
Weight of different vital rat organs was recorded
Fluorid e quantification by Atomic Absorption Spectroscopy (AAS) [Pending]
Organ index were calculated
Serial Dilution
Gut samples were homogenized
Culturable Gut Prokaryotes were Isolated

Figure 1: Graphical Representation of the Experimental Workflow

Keywords: Fluoride; Wistar rats; Sodium fluoride (NaF); Gut Microbiota; Dysbiosis; Health Risk; Groundwater; Antibiotic-resistance; Multiple Antibiotic Resistance (MAR) Index

Abbreviations: NaF-Sodium Fluoride; MAR-Multiple Antibiotic Resistance; CFU-Colony Forming Unit; MHA-Mueller-Hinton Agar; PCR-Polymerase Chain Reaction

INTRODUCTION

GUT MICROBIOTA AND THEIR ROLE IN HUMAN HOST HEALTH

The term microbiota refers to the wide range of microorganisms including bacteria, archaea, and fungi that live inside the human body, especially in the lower parts of the digestive system(Sender et al., 2016). These microbial communities have co-evolved with humans and play a crucial role in maintaining health (Claus et al., 2016). When the genetic information of these microbes is considered along with human DNA, the complete set is known as the microbiome. Gut colonization by these microbes begins before birth and continues to develop throughout a person's life (Qin et al., 2010). Several factors such as age, diet, health status, antibiotic use, and environment affect the makeup of the gut microbiota (Tamburini et al., 2016). The gut is predominantly inhabited by bacterial phyla such as Firmicutes, Bacteroidetes, and Proteobacteria, along with viruses (mainly bacteriophages) and fungi like Mycobacteria (Human Microbiome Project Consortium, 2012; Sender et al., 2016). Due to its significant role in digestion, metabolism, immunity, and mental health, the gut microbiota is often considered a "hidden organ." The interaction between humans and their gut microbes is generally beneficial or neutral (O'Hara & Shanahan, 2006). Though relatively stable in adults, the composition of the gut microbiota can be altered by factors such as genetics, dietary habits, medications, illnesses, inorganic pollutants and environmental pollutants like heavy metals potentially leading to health problems (Claus et al., 2016; Roca-Saavedra et al., 2018).

Gut microbes also serve as a primary line of defense against pathogenic organisms through a process known as "colonization resistance" (Buffie & Pamer, 2013). This process involves the competition between commensal bacteria (the beneficial microbes) and harmful pathogens for nutrients, attachment sites, and space within the gut (Kamada et al., 2013). The microbiota produces antimicrobial substances like bacteriocins and short-chain fatty acids (SCFAs), which inhibit the growth of pathogenic bacteria (Fukuda et al., 2011). Moreover, these beneficial microbes help strengthen the intestinal epithelial barrier, preventing the translocation of harmful pathogens into the bloodstream, which could otherwise lead to systemic infections (Martens et al., 2018).

The gut microbiota is essential for maintaining the integrity of the gut barrier, which is a physical and immune defense mechanism against pathogen invasion (Chelakkot et al., 2018). Tight junction proteins that hold the intestinal epithelial cells together form the gut’s first line of defense (Turner, 2009). A healthy microbiota supports the function of these tight junctions, helping to prevent gut permeability (also known as "leaky gut"). On the other hand, an imbalance in the microbiota (dysbiosis) can weaken the intestinal barrier, making it easier for pathogens to breach and cause systemic infections, inflammation, and even chronic conditions like obesity, diabetes, and cardiovascular diseases (Bischoff et al., 2014). Several factors can influence the composition and function of the gut microbiota, with diet being one of the most significant (Cani et al., 2008).

Diet is a major determinant of microbiota composition and function. Diets high in dietary fiber, fermented foods, and prebiotics foster microbial diversity and promote a beneficial microbial environment (Makki et al., 2018). Conversely, high-fat, low-fiber, and ultra-processed diets are linked to microbial imbalance and increased intestinal permeability (Devkota et al., 2012). Other lifestyle-related factors such as chronic stress, insufficient sleep, and sedentary behavior also negatively affect the gut microbiota and its immune-modulatory capacities (Zhu et al., 2022).

Research utilizing germ-free animal models has significantly underscored the essential role that gut microbiota play in supporting host physiology and health. Animals raised in sterile, microbe-free environments commonly exhibit developmental and metabolic abnormalities, primarily due to the absence of beneficial microbial interactions (Smith et al., 2007). Conversely, the symbiotic microbes residing in the human gastrointestinal tract contribute a variety of metabolic and biosynthetic functions that are critical for host survival. These microbial communities enhance nutrient absorption and are involved in synthesizing key biomolecules, such as vitamins and amino acids (Nicholson et al., 2012).

A wide range of factors can disrupt the balance of the gut microbiota, including genetics, diet, environmental exposures (such as heavy metals), medications, disease states, and lifestyle behaviors (Schirmer et al., 2016; Bischoff et al., 2014). These disruptions can impair the gut microbiota's critical role in modulating the immune system. Remarkably, nearly 80% of the body's total antibody production occurs in the gut-associated lymphoid tissue (GALT), with immunoglobulin A (IgA) as the predominant antibody isotype (Macpherson et al., 2008). Commensal microbes influence immune function by initiating regulatory mechanisms that limit inappropriate inflammation. They modulate multiple immune pathways, including suppression of Th1 and Th2 responses, reduction in IgE and IgG levels, and inhibition of Th17-associated inflammation (Belkaid & Hand, 2014).

Further evidence from germ-free mouse models highlights the essential role of gut microbes in immune development. These animals display poorly developed immune organs, significantly lower immunoglobulin levels, and an inability to induce oral tolerance to harmless dietary antigens like ovalbumin (Round & Mazmanian, 2009). Moreover, these mice lack proper regulatory T cell (Treg)-mediated responses, compromising immune homeostasis. Beyond their immunological impact, gut microbiota also supports intestinal epithelial development and regulate neuroendocrine signaling, thereby contributing to systemic health and homeostasis (O'Mahony et al., 2015).

In conclusion, preserving a diverse and balanced gut microbiota is fundamental to sustaining optimal health and immune functionality. The gut microbiota aids in immune regulation, supports pathogen defense mechanisms, and maintains the structural integrity of the intestinal barrier, all of which are vital in preventing disease (Belkaid & Hand, 2014; Clemente et al., 2012). To support microbial health, lifestyle factors such as a fiber-rich diet, regular physical activity, stress management, and judicious use of antibiotics are essential (Valdes et al., 2018). Prioritizing these

elements can help nurture a resilient gut ecosystem that enhances overall well-being and disease resistance.

ROLE OF GUT BACTERIAL COMPOSITIONAL BALANCE ON PHYSIOLOGY OF HOST

Microbial colonization of the human gut initiates at birth, although some studies suggest that limited microbial exposure may occur in utero (Perez-Muñoz et al., 2017). Several factors influence the composition and development of an infant's gut microbiota, including mode of delivery, gestational age, early feeding practices, and antibiotic exposure (Tamburini et al., 2016). Vaginally delivered infants typically acquire bacterial communities resembling their mother's vaginal microbiota, whereas those born via caesarean section are more likely to be colonized by skin-associated and environmental bacteria (Dominguez-Bello et al., 2010). Premature infants often have reduced populations of beneficial anaerobes such as Bacteroides and Bifidobacteria, which can affect early immune development (Arrieta et al., 2014). Additionally, the administration of antibiotics to the mother before or during delivery can alter the microbial seeding process, potentially delaying or disrupting normal microbiota development in the neonate (Francino, 2016).

The human gut microbiota is not limited to bacteria but also includes other microorganisms such as yeasts, protists, and viruses. The virome, particularly composed of bacteriophages, plays a significant role in shaping the gut ecosystem by regulating bacterial population dynamics and facilitating horizontal gene transfer (Manrique et al., 2017). Despite their importance, a substantial portion of viral sequences detected in the gut do not match any known references in current databases, making viral taxonomy and function challenging to decipher (Shkoporov & Hill, 2019). Fungal communities, primarily yeasts, form a relatively low-diversity population in the healthy adult gut, with fewer than 20 species typically detected (Hallen-Adams & Suhr, 2017). Although yeasts are present at much lower abundances than bacteria—by about four to five orders of magnitude—they possess significantly larger cell sizes and genomes, contributing unique metabolic and ecological functions to the overall microbiome (Huffnagle & Noverr, 2013).

Although every individual possesses a distinct composition of gut microbiota, research has identified consistent clustering patterns known as enterotypes. These enterotypes represent stable microbial communities structured through cooperative interactions among various microbial taxa, particularly through shared metabolic functions and ecological relationships (Arumugam et al., 2011). Such microbial interdependence promotes ecosystem resilience and supports the restoration of gut balance following disruptions.

As individuals age, however, the gut microbiota undergoes significant alterations. These age-related changes are characterized by a reduction in microbial diversity and notable shifts in the relative abundance of key bacterial groups (O'Toole & Jeffery, 2015). Such microbial shifts have been associated with inflammaging, a state of chronic, low-grade inflammation often observed in the elderly, resulting from a weakened and dysregulated immune response (Franceschi et al., 2018). Modulating the gut microbiota—either through dietary interventions, probiotics, or

microbial transplantation—has shown potential in mitigating some of the physiological declines linked to ageing (Biagi et al., 2016).

FACTORS ALTERING THE STEADY STATE OF GUT MICROBIOTA

The **steady-state of the gut microbiota** denotes a condition in which the microbial community within the gastrointestinal tract remains balanced and resilient, functioning optimally to support host health. This equilibrium is essential for efficient digestion, nutrient uptake, immune homeostasis, and defense against invading pathogens (Sommer & Bäckhed, 2013). However, a variety of intrinsic and extrinsic factors—such as diet, infections, antibiotics, stress, and environmental exposures—can disturb this balance, leading to a condition known as **gut dysbiosis** (Valdes et al., 2018). Dysbiosis, or microbial imbalance, has been implicated in numerous health conditions, including inflammatory bowel diseases, metabolic disorders like obesity and type 2 diabetes, autoimmune diseases, allergic reactions, and neuropsychiatric disorders such as anxiety and depression (Rinninella et al., 2019; Cryan et al., 2019). Restoring gut microbial balance through dietary interventions, probiotics, prebiotics, or fecal microbiota transplantation has thus become a growing area of therapeutic interest.

- **Dietary Patterns:** Diet plays a central role in shaping the composition and function of the gut microbiota, as it supplies nutrients not only to the host but also to resident microbial communities. Humans are unable to digest certain complex plant-derived carbohydrates due to the absence of specific enzymes; however, gut microbes compensate by fermenting these compounds into beneficial metabolites (Flint et al., 2012). It is estimated that approximately 20–60 grams of undigested carbohydrates reach the colon daily, where they serve as substrates for microbial fermentation (Scott et al., 2013). This fermentation process yields short-chain fatty acids and other compounds that influence host physiology. Because various bacterial taxa specialize in metabolizing distinct nutrients, dietary composition directly affects the microbial population and its metabolic outputs, thereby impacting overall gut and systemic health (David et al., 2014).
- **Aging:** The development of the human gut microbiota is a dynamic and lifelong process, with microbial diversity shifting at different stages of life. Colonization begins at birth, when the infant's gastrointestinal tract is first populated by microorganisms. This early microbial community is generally unstable, with frequent fluctuations in composition (Arrieta et al., 2014). In vaginally delivered infants, initial exposure occurs during passage through the birth canal, where maternal vaginal and intestinal microbes—such as *Lactobacillus* and *Bifidobacterium* species—are transferred to the newborn (Dominguez-Bello et al., 2010). Conversely, infants delivered via caesarean section encounter microbes from the surrounding environment, including those present on medical staff and in hospital settings. This often results in lower microbial load and reduced diversity during the early postnatal period (Biasucci et al., 2008; Jakobsson et al., 2014).

- **Antibiotic Use:** Antibiotic treatment can significantly disrupt gut microbiota, often lowering its diversity and impairing its normal functions. While recovery may occur within weeks, some individuals experience long-term shifts, with beneficial microbes failing to return (Dethlefsen & Relman, 2011). These changes can alter metabolism, bile acid processing, and hormone regulation (Palleja et al., 2018). In infants, early antibiotic exposure can disturb microbial colonization, potentially affecting immune development (Yassour et al., 2016).
- **Environmental Influences:** Limited microbial exposure in early life—often due to overly sterile environments—can impair immune tolerance and increase the risk of allergies and autoimmune disorders (Rook, 2012). Additionally, chronic exposure to environmental toxins and heavy metals may disrupt gut integrity, increase intestinal permeability, and trigger systemic inflammation (Zhao et al., 2020).
- **Stress and Mental Health:** Psychological stress affects gut health via the gut-brain axis, disrupting microbial balance through stress hormones like cortisol and norepinephrine (Foster et al., 2017). These changes can reduce beneficial microbes, impair neurotransmitter production (e.g., serotonin, GABA), and are associated with mood disorders and anxiety (Cryan et al., 2019).
- **Obesity:** Obesity has been linked to chronic low-grade inflammation, partially driven by gut microbiota-derived compounds such as lipopolysaccharides (LPS). This translocation is often facilitated by high-fat diets and increased gut permeability seen in obesity (Cani et al., 2007). Elevated LPS levels can promote metabolic imbalances, contribute to insulin resistance, and support fat storage, thereby establishing a connection between microbial activity and obesity-related metabolic disorders (Cani & Delzenne, 2009).
- **Lifestyle choices:** Lifestyle behaviours significantly influence the composition and function of the gut microbiota. Unhealthy habits such as smoking, heavy alcohol intake, insufficient sleep, and physical inactivity can disrupt microbial equilibrium, often decreasing beneficial microbes and encouraging the growth of potentially pathogenic species (Valdes et al., 2018). Such dysbiosis has been linked to systemic inflammation, compromised immunity, and gastrointestinal issues. Conversely, maintaining regular exercise, getting quality sleep, and making health-conscious choices can promote microbial diversity and support better gut function. These positive behaviours enhance immune regulation and metabolic health, contributing to a balanced and resilient gut ecosystem (Mailing et al., 2019).

- **Health Conditions and Treatments:** Chronic illnesses such as Inflammatory Bowel Disease (IBD) are both influenced by and contribute to gut microbial imbalance. Individuals with IBD typically exhibit a reduction in beneficial bacteria and an overgrowth of harmful species, including *Escherichia coli* (Ni et al., 2017). Similarly, conditions like obesity, insulin resistance, and type 2 diabetes are associated with dysbiosis, which can affect microbial metabolic activity and trigger systemic inflammation (Tilg et al., 2020). Furthermore, cancer therapies often disrupt the gut microbiome, leading to increased gastrointestinal complications, higher infection risk, and diminished therapeutic outcomes (Alexander et al., 2017).

IMPACT OF DIFFERENT ENVIRONMENTAL POLLUTANT AND OTHER FACTORS ON GUT DYSBIOSIS

Environmental pollutants have a profound influence on gut microbial communities in animals, often resulting in dysbiosis—an imbalance in microbial composition that disrupts normal physiological functions. Exposure to substances like heavy metals, pesticides, and synthetic additives can diminish beneficial bacterial populations while promoting the growth of harmful microbes (Sharma et al., 2020). These shifts can alter vital microbial processes such as the synthesis of short-chain fatty acids (SCFAs), amino acids, and other metabolites crucial for host health (Rico et al., 2021). Furthermore, xenobiotics can directly or indirectly induce microbiome toxicity, promoting the emergence of resistant strains and compromising microbial diversity (Claus et al., 2016). The chronic intake of these environmental contaminants underlines the urgent need for stricter regulations and increased public awareness to preserve gut microbial balance and prevent disease development.

Effects of environmental chemical exposure on the gut microbiota:

- **Heavy Metals:** Heavy metals are classified as either essential—such as iron, copper, and zinc—or toxic, including lead, cadmium, and arsenic. Essential metals support vital metabolic and immune functions by fostering beneficial gut microbes and aiding enzymatic processes (Tchounwou et al., 2012). Conversely, toxic metals can induce oxidative stress, disrupt enzyme activities, and cause cellular injury, often through interactions with gut microbiota. Chronic exposure to these harmful metals has been associated with neurological disorders, cancer development, and disruptions to the gut-brain axis (Jomova & Valko, 2011; Gao et al., 2020). Lead, arsenic, and cadmium are notable toxic metals negatively influencing gut microbial balance and intestinal health.

HEAVY METALS	SOURCES	PROPERTIES	MECHANISM OF ACTION	HEALTH IMPLICATIONS
LEAD (Pb)	Lead-based paints, old plumbing systems, Battery production, Soil contamination	Highly toxic, Builds up in bones and tissues, No safe threshold	Damages tight junctions in the gut, stimulates immune response in the mucosa, Affects gut-brain communication	Linked to behavioural disorders like ADHD, promotes systemic inflammation, Increases risk of metabolic conditions
ARSENIC (As)	Contaminated groundwater, Rice and seafood Industrial by-	Occurs in organic and inorganic forms, Inorganic arsenic is highly toxic,	Disrupts microbial metabolism in gut, Hampers detoxification pathways, alters	Promotes colon and liver cancer, induces insulin resistance and diabetes, weakens immune defense,

	products, Treated wood	Carcinogenic (IARC Group 1)	immune signalling, Encourages microbial imbalance	Causes chronic gut inflammation
CADMIUM (Cd)	Industrial emissions, Phosphate fertilizers, Tobacco smoke, Contaminated grains & leafy vegetables	Non-essential and toxic, long biological half-life (10–30 years), Accumulates in kidneys and liver	Induces oxidative stress in gut microbiota, weakens gut lining, Alters SCFA synthesis, lowers mucosal immunity, Increases intestinal permeability	Associated with IBD and colorectal cancer, impairs nutrient uptake, may disrupt neurodevelopment via gut-brain axis
MERCURY (Hg)	Seafood (methylmercury), dental amalgams, coal combustion, mining and industrial waste	Highly neurotoxic (especially methylmercury), Fat-soluble and accumulative, Crosses blood-brain and placental barriers	Disrupts microbial DNA and enzyme activity, elevates inflammatory cytokines, Impairs gut-brain axis signalling	May contribute to autism spectrum conditions, causes neuroinflammation and oxidative stress, Leads to digestive system issues
CHROMIUM (Cr)	Leather processing, Electroplating, Dyes and pigments, Steel industries	Cr (III): essential trace element, Cr (VI): highly toxic and cancer causing, Water soluble and motile	Causes apoptosis in gut lining cells, enhances intestinal leakiness, generates reactive oxygen species (ROS), Suppresses anti-inflammatory responses	May result in colorectal cancer, leads to gut inflammation, heightens vulnerability to infections, Disrupts glucose regulation
NICKEL (Ni)	Stainless steel, Jewelry, Batteries, Industrial waste, Contaminated food and water	Trace element but toxic in excess, Can bioaccumulate, Allergenic and pro-inflammatory	Alters gut microbial balance, disrupts epithelial barrier, increases oxidative stress, Impairs microbial enzyme activity	Associated with allergic reactions and dermatitis, can aggravate asthma, contributes to inflammation and gut permeability, May be linked to autoimmune issues and metabolic dysfunctions

Table 1: Some Common Heavy Metals

- **Phthalates:** Phthalates, which are widely present in plastic materials and consumer products, have been linked to changes in the gut microbiota. These chemical compounds can shift the diversity and composition of gut bacteria and influence microbial gene expression. Such disturbances may lead to health concerns like metabolic dysfunction and immune imbalance (Zhou et al., 2017; Wang et al., 2020).
- **Pesticides:** Pesticides, frequently applied in agriculture for pest control, have been shown to negatively affect gut microbial populations. These compounds can modify the composition and gene expression of gut microbes, disturbing normal metabolic activities within the microbiome. As a result, individuals exposed to pesticides may face risks of gut-related diseases and immune system impairment (Rueda-Ruzafa et al., 2019; Mesnage & Antoniou, 2017). Human exposure mostly comes from contaminated food and drinking water. While excess intake is a concern, dietary nitrates can also offer health benefits by improving vascular function, reducing inflammation, and enhancing immune responses through increased neutrophil activity. These benefits may lower risks of cardiovascular and metabolic conditions in animals and humans (Kapil et al., 2010; Hord et al., 2009).
- **Microplastics:** Microplastics—tiny plastic particles commonly found in food and water—can damage the gut lining, trigger inflammation, and disturb the balance of gut microbes. This dysbiosis may impair digestion and immune function. Moreover, microplastics often carry toxic substances such as heavy metals or organic pollutants, intensifying gut-related harm and increasing long-term risks for metabolic and inflammatory disorders (Jin et al., 2019; Wang et al., 2021).
- **Endocrine Disruption:** Endocrine-disrupting chemicals (EDCs), including some pesticides and plastic components, interfere with hormone signaling and can impact gut microbiota. Hormones such as estrogen and glucocorticoids regulate digestion and immunity, so EDC exposure may cause dysbiosis, inflammation, and higher susceptibility to diseases like obesity, diabetes, and autoimmune conditions (Zhou et al., 2021; Tait et al., 2020).
- **Persistent Organic Pollutants (POPs):** Persistent Organic Pollutants (POPs) are long-lasting, fat-soluble chemicals that resist environmental and microbial degradation. Due to their lipophilic nature, they bioaccumulate in the food chain and are mainly ingested through high-fat foods. POPs, such as polychlorinated biphenyls (PCBs), are associated with endocrine disruption, neurotoxicity, and genotoxic effects. Infants are especially vulnerable, as PCBs can transfer through breast milk. The high halogen content of POPs contributes to their persistence and long-term health risks (Lee et al., 2017; Wania & Mackay, 1996; La Merrill et al., 2020).
- **Inorganic Pollutant:** Inorganic pollutants are chemical substances that do not contain carbon and can pollute the environment. **Fluoride i**s an inorganic pollutant naturally found in soil, water, and air, and although it plays an essential role in promoting dental health and bone strength when consumed in trace amounts, chronic overexposure can lead to toxic effects. Prolonged ingestion of high levels of fluoride can disrupt the body's mineral homeostasis, particularly interfering with calcium and phosphorus metabolism, and may result in dental and skeletal fluorosis (World Health Organization [WHO], 2011). Research indicates that elevated fluoride intake can damage multiple organs including the liver, kidneys, lungs, and

reproductive tissues such as the testes (Wang et al., 2021). Additionally, high fluoride exposure has been associated with neurotoxic effects, such as reduced intelligence in children and a potential increased risk of bone cancers (Choi et al., 2012; Bassin et al., 2006).

Drinking water remains the primary route of fluoride exposure, prompting WHO to recommend maintaining fluoride concentrations between 0.5 and 1.5 mg/L to safeguard human health (WHO, 2011). In certain regions of China, particularly where brick tea containing high fluoride levels is widely consumed, increased cases of skeletal and dental fluorosis have been documented, underscoring a significant public health issue (Wei et al., 2023). Fluoride has also been shown to negatively affect intestinal epithelial cells by compromising gut barrier integrity and reducing cellular viability. One prominent toxicological mechanism involves the induction of oxidative stress, wherein excess reactive oxygen species (ROS) overwhelm antioxidant defenses, leading to lipid peroxidation and a form of programmed cell death known as ferroptosis (Tang et al., 2022; Li et al., 2023).

Animal studies further highlight that chronic fluoride exposure can significantly alter the gut microbiome, affecting both the richness and diversity of microbial communities in the ileum and colon (Gao et al., 2023). While fluoride is essential in small amounts, environmental sources such as industrial emissions, phosphate fertilizers, and wastewater discharge can result in excessive accumulation, posing ecological and human health risks. Consequently, excessive fluoride is classified as a key environmental contaminant with the potential to cause systemic toxicity and microbial dysbiosis (Fluegge, 2016; WHO, 2011).

- **Antibiotics:** Antibiotics have significantly transformed the treatment of bacterial infections, contributing to major improvements in public health. However, their widespread and sometimes excessive use has raised concerns regarding their unintended effects on the gut microbiota, which plays a crucial role in maintaining overall health (Francino, 2016). Antibiotics function by inhibiting essential bacterial processes such as cell wall synthesis, protein production, and DNA replication. While these mechanisms effectively eliminate pathogenic bacteria, they also harm beneficial commensal microbes, particularly genera like Bifidobacterium and Lactobacillus (Palleja et al., 2018). These probiotic species are vital for breaking down dietary fibers, synthesizing vitamins, enhancing immune responses, and protecting against pathogenic invasion. One of the most severe consequences of such microbial imbalance is C. difficile infection (CDI), which often manifests as persistent diarrhea and colonic inflammation, particularly following antibiotic treatment (Buffie & Pamer, 2013). Beyond acute infections, prolonged or repeated antibiotic exposure has been associated with the onset and exacerbation of chronic gastrointestinal conditions like inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis. These conditions are believed to result from a combination of reduced microbial diversity, compromised intestinal barrier function, and dysregulated immune responses (Ni et al., 2017). Moreover, frequent antibiotic usage facilitates the emergence of antibiotic resistance genes (ARGs) within the gut microbiome. These genes can be horizontally transferred among microbes, making future infections more challenging to treat and contributing to the global public health threat of antimicrobial resistance (Buelow et al., 2020; Modi et al., 2014).

ROOT OF ANTIBIOTIC RESISTANCE AND ITS IMPACT ON GUT DYSBIOSIS

Antibiotics are antimicrobial agents that combat bacterial infections by either killing bacteria (bactericidal) or inhibiting their growth (bacteriostatic). Their pharmacological action involves disrupting bacterial cell processes essential for survival or reproduction (Walsh, 2003). Antibiotics are typically classified based on their in vitro effects: bactericidal agents directly kill bacteria, whereas bacteriostatic agents prevent bacterial proliferation (Lorian, 2005). However, the widespread and often excessive use of antibiotics has contributed significantly to the emergence of antibiotic resistance, which compromises the effectiveness of these drugs and disrupts the gut microbiota (Ventola, 2015). Antibiotic resistance occurs when bacteria undergo genetic changes, either spontaneously or via horizontal gene transfer, enabling them to survive previously lethal antibiotics (Davies & Davies, 2010). This resistance leads to uncontrolled bacterial growth, complicating treatment and increasing the likelihood of recurrent infections (World Health Organization, 2020).

Antibiotic resistance arises when bacteria develop mechanisms to evade the effects of antibiotics. These mechanisms include producing enzymes such as β-lactamases, modifying antibiotic target sites, employing efflux pumps to expel drugs, reducing membrane permeability, and forming protective biofilms (Munita & Arias, 2016). Resistance genes are often transferred rapidly via mobile genetic elements like plasmids and transposons (Partridge et al., 2018). The misuse of antibiotics in clinical settings and their overuse in agriculture significantly drive resistance development (O'Neill, 2016). Additionally, environmental contamination from pharmaceutical waste and improper drug disposal facilitates the dissemination of resistance genes through ecosystems including soil and water (Larsson et al., 2018). Horizontal gene transfer through conjugation, transformation, and transduction further spreads these resistance traits among bacteria (Thomas & Nielsen, 2005).

While antibiotics are critical for treating bacterial infections, they can disrupt the gut microbiota by eliminating beneficial microbes along with pathogens, causing dysbiosis (Dethlefsen et al., 2008). This imbalance is associated with inflammation, impaired immunity, and conditions such as obesity, diabetes, and neuropsychiatric disorders (Llorente & Schnabl, 2016). The reduction in colonization resistance renders the gut more susceptible to opportunistic infections (Buffie & Pamer, 2013). Prolonged or inappropriate antibiotic use decreases microbial diversity and promotes the proliferation of antibiotic resistance genes (ARGs) in the gut microbiome, which may be transferred to pathogenic bacteria, complicating treatment (Penders et al., 2013). The degree of microbiota disruption depends on the antibiotic type, dosage, and treatment duration, potentially leading to persistent issues like recurrent infections and antibiotic-associated diarrhea (Jernberg et al., 2010).

ROLE OF DIFFERENT ENVIRONMENTAL POLLUTANTS IN THE EMERGENCE AND SPREAD OF ANTIBIOTIC RESISTANCE

Environmental pollution is increasingly recognized as a major driver of antibiotic resistance, alongside antibiotic misuse in healthcare (Martínez, 2012). Contaminants released into air, soil, and water create selective pressures on microbial communities that foster resistance development even without direct antibiotic exposure (Karkman et al., 2018). Agricultural chemicals like glyphosate and chlorpyrifos disrupt bacterial membranes and stress responses, promoting genetic mutations and horizontal gene transfer of resistance determinants (Kurenbach et al., 2015). Non-antibiotic drugs, including anti-inflammatory agents and disinfectants, may induce cross-resistance via shared regulatory pathways (Pal et al., 2015). Wastewater treatment plants (WWTPs) are hotspots for resistance emergence and dissemination because of diverse microbial populations and pollutant mixtures, with treated effluent still releasing resistant bacteria and genes into ecosystems (Rizzo et al., 2013). Microplastics provide surfaces for biofilm formation—known as the "plastisphere"—facilitating genetic exchange and ARG persistence (Kirstein et al., 2016). Heavy metals such as lead, arsenic, mercury, and copper, commonly found in industrial and agricultural waste, co-select for antibiotic resistance as resistance genes are often linked on mobile genetic elements (Seiler & Berendonk, 2012).

Environmental pressures significantly promote the survival and proliferation of antibiotic-resistant bacteria. Pollutants induce oxidative stress in bacterial cells, increasing mutation and recombination rates that facilitate the emergence of resistance (Gill & Flint, 2011). Many resistance genes reside on mobile genetic elements such as plasmids, transposons, and integrons, which enable their efficient transfer across bacterial communities (Domingues et al., 2012). Consequently, environmental reservoirs act as critical hubs for the maintenance, evolution, and dissemination of antibiotic resistance genes (ARGs) (Wellington et al., 2013).

EFFECT OF GUT DYSBIOSIS ON HUMAN HEALTH

1. **Gastrointestinal Disorders:** GI disorders affect the digestive organs, impairing digestion and nutrient uptake. Conditions like Irritable Bowel Syndrome (IBS) cause abdominal discomfort and irregular bowel habits, while Inflammatory Bowel Disease (IBD)—including Crohn's disease and ulcerative colitis—is characterized by chronic inflammation and symptoms such as bloody diarrhea. Gut dysbiosis, marked by decreased beneficial bacteria like Firmicutes and increased harmful ones such as Enterobacteriaceae, is strongly associated with IBD (Lopez-Siles et al., 2018). Other common GI issues include gastritis, constipation, and diarrhea.
2. **Metabolic Disorders:** Metabolic diseases result from disrupted metabolism, with type 2 diabetes (T2D) and obesity being prevalent examples. T2D involves insulin resistance and high blood glucose, with gut microbiota playing a critical role influenced by early-life microbial exposure (Qin et al., 2012). Dysbiosis impairs insulin sensitivity and can trigger diabetes. Obesity correlates with an altered gut microbiome, notably an increased Firmicutes-to-

Bacteroidetes ratio, influenced by diet and lifestyle (Turnbaugh et al., 2006). Metabolic syndrome is also linked to microbial imbalances.

3. **Central Nervous System (CNS) Disorders**: The gut-brain axis connects gut microbiota to CNS function through neural, immune, and hormonal pathways. Dysbiosis can cause increased intestinal permeability ("leaky gut"), allowing endotoxins like lipopolysaccharides (LPS) to induce neuroinflammation, contributing to neurodegenerative diseases including Alzheimer's and Parkinson's (Cryan et al., 2019). It also disrupts neurotransmitter production (serotonin, GABA), influencing mental health conditions such as anxiety and depression. Autism Spectrum Disorder (ASD) shows associations with altered gut microbial profiles (Hsiao et al., 2013).
4. **Autoimmune Disorders:** Autoimmune diseases involve the immune system attacking self-tissues, driven by genetic and environmental factors including gut microbiota alterations. Rheumatoid arthritis (RA) causes joint inflammation and damage, affecting about 1% of people worldwide, predominantly women (Scher et al., 2013). Systemic lupus erythematosus (SLE) is characterized by widespread organ inflammation and is linked to microbial dysbiosis.
5. **Mental Health Disorders:** Disorders such as depression, anxiety, and ASD have been increasingly connected to gut microbial imbalances. Anxiety and major depressive disorder (MDD) display altered gut bacterial populations compared to healthy individuals (Kelly et al., 2016). ASD and other mental illnesses like bipolar disorder, PTSD, and schizophrenia also show microbiome involvement, making the gut microbiota a potential therapeutic target (Hsiao et al., 2013).
6. **Cardiovascular Disorders**: Emerging evidence links gut dysbiosis to cardiovascular diseases like atherosclerosis and coronary artery disease. Disrupted gut barrier integrity allows LPS to enter circulation, activating inflammatory pathways that contribute to plaque buildup (Wang et al., 2011). Elevated trimethylamine-N-oxide (TMAO) levels, decreased bile salt hydrolase-producing bacteria, and microbial DNA presence in plaques underscore microbial roles. Gut microbiota also affect blood pressure regulation, with distinct microbial signatures found in hypertensive patients, especially under high-salt diets (Li et al., 2017).

FLUORIDE AS AN INORGANIC POLLUTANT

Fluoride is an inorganic compound formed when fluorine, a highly reactive halogen, bonds with elements such as metals or hydrogen. It naturally occurs in minerals like fluorite (CaF_2) and cryolite (Na_3AlF_6), as well as in soil, rocks, and water (Whitford, 1996). At low concentrations, fluoride benefits human health by strengthening tooth enamel and preventing dental cavities (Fejerskov et al., 2015). Industrially, fluoride compounds are used in aluminum production, glass and ceramic manufacturing, and the synthesis of fluorinated chemicals and pesticides (Murray, 2012).

However, fluoride becomes problematic as an environmental pollutant when present in excessive amounts. Human activities such as coal burning, phosphate fertilizer manufacturing, industrial

effluents, and improper waste disposal release fluoride into ecosystems (Susheela, 2012). In some areas, natural fluoride-rich groundwater combined with overextraction worsens contamination (Singh et al., 2011). Elevated fluoride levels in water, soil, and air pose health risks including dental and skeletal fluorosis, which cause damage to teeth and bones after prolonged exposure (Ayoob & Gupta, 2006). Fluoride also disrupts metabolic functions, enzyme activity, and nutrient uptake in plants and animals, and alters microbial communities in soil and aquatic habitats, affecting ecosystem health and nutrient cycling (Camargo, 2003). Overall, fluoride has important natural and industrial roles, its excessive environmental presence due to anthropogenic sources makes it a persistent pollutant with significant ecological and health consequences.

SOURCES OF FLUORIDE

a) **Natural Sources** (Smedley et al., 2002):
   1. Groundwater – Especially in areas with fluoride-rich geological formations (e.g., volcanic rocks, granites).
   2. Rocks and Soil – Fluoride is naturally present in the Earth's crust and can leach into water.
   3. Seafood – Marine fish, shellfish, and seaweed tend to have higher natural fluoride levels.
   4. Tea Leaves – Tea plants accumulate fluoride from soil and air, making brewed tea a common source.
   5. Mineral Springs – Some natural mineral waters contain high fluoride concentrations.

b) **Anthropogenic Sources (**Akuno et al., 2016)**:**
   1. Fluoridated Drinking Water – In some countries, fluoride is added to public water supplies for dental health.
   2. Dental Products – Toothpaste, mouth rinses, and gels often contain fluoride.
   3. Food and Beverages – Processed foods and drinks prepared with fluoridated water.
   4. Pesticides and Fertilizers – Especially those used in agriculture that may contain fluoride compounds.
   5. Industrial Emissions – Industries such as aluminum smelting, brick-making, and phosphate fertilizer production release fluoride into air and water.
   6. Pharmaceuticals – Some medications and anesthetics (e.g., sevoflurane) contain fluoride.
   7. Teflon and Other Fluoropolymers – When degraded, they can release fluoride compounds into the environment.

FLUORIDE CONTAMINATION IN GROUNDWATER

Approximately 85% of India's population relies on groundwater for drinking purposes, making water quality a major public health issue (World Bank, 2012). Traditionally, groundwater in India was considered largely safe and was extensively used without much scrutiny. However, recent

studies have revealed both geogenic and anthropogenic contaminants in groundwater, raising significant health concerns (Shekhar & Sarkar, 2013; Sarkar et al., 2016; Saha et al., 2016; Kumar et al., 2017). Among these contaminants, fluoride pollution stands out due to its persistent nature and adverse health impacts, particularly dental and skeletal fluorosis, which also contribute to social and economic burdens (Ali et al., 2016).

Prolonged rock-water interaction, longer residence time, and evapotranspiration processes can elevate fluoride concentrations in aquifers (Dhadge et al., 2018). Although not universal, deeper aquifers tend to have higher fluoride levels. Groundwater with elevated fluoride typically exhibits neutral to alkaline pH, elevated sodium and bicarbonate, and lower calcium concentrations—conditions that enhance the dissolution of fluoride from minerals (Ali et al., 2016). The alkaline pH range (7.6–8.6) is particularly conducive to fluoride mobilization, and such waters often demonstrate a positive correlation between fluoride, sodium, and bicarbonate, with calcium concentrations inversely related (Shekhar, 2017).

Fluoride contamination has received increasing attention due to its bioaccumulative and non-biodegradable nature, posing long-term health hazards (Saxena & Ahmed, 2001; Ali et al., 2016). Both natural processes and human activities contribute to elevated fluoride levels in drinking water, making it unsafe for human consumption. The primary geogenic factors responsible include fluoride-rich aquifers, regional geology, weathering of minerals, hydrochemical reactions, and contaminant migration through subsurface pathways (Daneshpooy et al., 2018; Yousefi et al., 2018a).

Chronic exposure to high fluoride levels can cause fluorosis, which affects not only humans but also livestock. It is estimated that over 66 million individuals in India are at risk of fluoride-induced health disorders (Jacks et al., 2005; Ali et al., 2016). Given these impacts, it is imperative to evaluate the interplay of geochemical, hydrogeological, climatic, and anthropogenic factors to better manage and mitigate fluoride contamination (Saxena & Ahmed, 2001; Yousefi et al., 2018b).

IMPACT OF FLUORIDE ON GUT MICROBES

Fluoride, although beneficial in small amounts—particularly for **preventing dental caries**—can adversely affect the **gut microbiota** when consumed in excessive quantities. The gut microbiota is a **highly diverse and dynamic ecosystem** that plays essential roles in digestion, immune system modulation, nutrient absorption, and the maintenance of metabolic and intestinal homeostasis (Gomaa, 2020; Lynch & Pedersen, 2016). However, elevated fluoride exposure, especially from **contaminated drinking water or food**, can disturb this microbial equilibrium, leading to **gut dysbiosis**—a condition characterized by the loss of beneficial microbial diversity and an increase in harmful bacteria (Zhang et al., 2021).

Fluoride exhibits **broad-spectrum antimicrobial activity**, which, while useful against cariogenic oral bacteria, may also inhibit **commensal and probiotic gut microbes**. Research has indicated that fluoride exposure leads to a **reduction in microbial diversity** and suppresses populations of beneficial bacteria such as *Lactobacillus*, *Bifidobacterium*, and *Clostridium* species (Chen et al., 2020; Li et al., 2021). Concurrently, there may be a **relative enrichment of pro-inflammatory and potentially pathogenic bacteria**, thereby altering the gut microbial structure and function (Zhang et al., 2021).

This dysbiosis negatively impacts the production of short-chain fatty acids (SCFAs)—key microbial metabolites like acetate, propionate, and butyrate—that are essential for colonocyte health, intestinal barrier integrity, and immune modulation (Koh et al., 2016). A disrupted gut environment due to excessive fluoride may lead to increased intestinal permeability ("leaky gut"), impaired mucosal immunity, and heightened systemic inflammation (Zhao et al., 2021). Such physiological alterations have been linked to a wide array of health conditions including metabolic syndrome, autoimmune disorders, neurodevelopmental abnormalities, and inflammatory bowel diseases (Zhang et al., 2021; Chen et al., 2020). Thus, while fluoride serves as a valuable preventive measure in oral healthcare, uncontrolled or chronic exposure may compromise gut health, emphasizing the importance of monitoring environmental fluoride levels, particularly in endemic regions.

REVIEW OF LITERATURE

Fluoride is widely used in public health due to its well-established role in preventing tooth decay. However, concerns have grown regarding its potential adverse effects on human health when consumed in excessive amounts, particularly through contaminated groundwater. One area of increasing interest is its impact on the gut microbiota, a complex community of trillions of microorganisms essential for digestion, immunity, metabolism, and overall homeostasis (Pal et al., 2014; Banerjee et al., 2016). Disruption of this microbial balance—known as gut dysbiosis—due to toxic agents like fluoride may lead to various metabolic and immunological disorders (Mukherjee et al., 2017; Sharma et al., 2019; Sakthi Desai et al., 2020).

Recent studies have begun to explore how fluoride affects the structure and function of gut microbial communities, as well as the possibility of fluoride-resistant bacterial strains in different environments. For example, research in Tamil Nadu, India, identified fluoride-tolerant bacterial strains such as Enterobacter and Aeromonas that could survive in fluoride concentrations as high as 300 mM. Genetic analyses revealed the presence of fluoride resistance genes, which may contribute to their ability to extrude fluoride ions and mitigate toxicity (Kirupa Sree et al., 2018; Edward Raja et al., 2021; Baker et al., 2012). Although such studies have primarily focused on environmental samples, these findings prompt further investigation into whether similar fluoride resistance mechanisms exist in gut-residing microorganisms.

Animal models also provide insight into the microbiological effects of fluoride. Rodent-based research has shown that fluoride intake leads to shifts in microbial composition, often marked by a decline in bacterial diversity and alterations in the ratio of Firmicutes to Bacteroidetes, two dominant phyla in the gut. These shifts were associated with increased inflammation and reduced short-chain fatty acid (SCFA) production, crucial for gut barrier integrity and immune modulation (Viswanathan et al., 2010; Selvam et al., 2016; Amalraj & Pius, 2018).

It has also been hypothesized that long-term exposure to fluoride can create selective pressure within the gut, promoting the survival and proliferation of resistant bacterial strains (Amini et al., 2008; Thivya et al., 2017). This is particularly relevant in fluoride-endemic areas where drinking water contains naturally high fluoride levels. However, the majority of current studies rely on faecal samples, which may not fully capture the microbial diversity of the entire gastrointestinal tract (Subba Rao, 2017). Additionally, there is a lack of comprehensive in vivo studies that isolate and characterize fluoride-resistant gut bacteria directly from mammalian hosts.

Further research is necessary to identify these fluoride-resistant strains in the gut and investigate their adaptation mechanisms, which may include genetic mutations, altered membrane transport systems, or the use of fluoride-specific efflux pumps. These studies could offer valuable insights into microbial resilience and the broader health implications of chronic fluoride exposure (Adimalla et al., 2019; Amalraj & Pius, 2018).

NATIONAL AND INTERNATIONAL STATUS

Fluoride exposure has been widely studied both internationally and in India, highlighting its dual role beneficial in small amounts for dental health, but harmful when consumed excessively. Fluoride is well known for its effectiveness in preventing tooth decay when maintained at optimal levels(WHO, 2017; Chouhan & Flora, 2010).

Recent research has significantly advanced our understanding of bacterial fluoride resistance, particularly concerning its impact on gut microbiota and potential health implications. A pivotal study demonstrated that *Bifidobacterium* species can alleviate fluoride-induced toxicity in mice. The administration of these probiotics reduced pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in the serum, liver, and ileum. Moreover, *Bifidobacterium* intervention mitigated morphological damage in the duodenum, ileum, colon, and cecum, suggesting a protective role against fluoride-induced gastrointestinal injury (Cheng et al.,2024; Wang et al.,2024; Liang et al.,2024).

In environmental contexts, studies have isolated fluoride-resistant bacteria from contaminated water sources. These strains exhibited resistance to multiple antibiotics and harbored the *crcB* gene, known for its role in fluoride efflux. Similarly, *Pseudomonas aeruginosa* strains from water samples demonstrated high fluoride resistance and possessed virulence factors and biofilm-

forming genes, indicating potential health risks (Kirupa et al.,2018; Ramesh et al., 2018; Edward et al., 2018).

Further investigations into the gut microbiota have revealed that fluoride exposure can disrupt microbial balance. A study involving mice exposed to varying fluoride concentrations showed that high doses (10 and 15 mg/L) significantly altered gut microbiota composition, reducing beneficial bacteria like *Faecalibacterium* and increasing harmful taxa such as *Proteobacteria*. Another study found that fluoride exposure led to decreased goblet cell numbers and glycoprotein expression in the colon, along with significant changes in microbial diversity and composition. These alterations were associated with reduced short-chain fatty acid levels and increased expression of inflammatory markers (Guijie Chen et al.,2020; Pengcheng Hu et al.,2020; Zhichao Xu et al.,2020; Chuanyi Peng et al.,2020; Yijun Wang et al.,2020; Xiaochun Wan et al.,2020; Huimei Cai et al.,2020).

In the context of wastewater treatment, biofilms formed by fluoride-resistant bacteria like *Pseudomonas* and *Bacillus* species have been studied. These biofilms exhibit adaptations such as enhanced efflux pump activity and metabolic shifts to cope with fluoride stress. However, these changes can lead to reduced biodiversity and diminished metabolic functions, impacting the efficiency of wastewater treatment processes. Collectively, these studies underscore the adaptive capabilities of certain bacterial species to high-fluoride environments and highlight the potential consequences for human health, particularly concerning gut microbiota balance and the emergence of antibiotic-resistant strains. Continued research in this area is essential to develop strategies for mitigating fluoride toxicity and preserving microbial health (Zhong et al.,2024; Wang et al.,2024; Quan et al.,2024).

SCIENTIFIC RATIONALE OF THE STUDY

Fluoride is a common inorganic pollutant, especially in areas like India where high levels are often found in groundwater (Chouhan & Flora, 2010; Amini et al., 2008). While fluoride is known for its dental benefits in small amounts, excessive exposure can lead to health issues, including damage to bones, teeth, kidneys, and the liver. Although many studies have explored how fluoride affects human health, very few have looked at its impact on gut bacteria (WHO, 2017; Adimalla et al., 2019). There is also a growing concern that exposure to high fluoride levels not only disrupts gut microbial balance but may also contribute to the development of antibiotic-resistant bacteria by co-selecting for resistance traits (Chen et al., 2020; Wang et al., 2020). Most existing studies rely on faecal samples, which may not truly represent the gut's microbial diversity. There is a lack of in-depth in-vivo studies focused on isolating and identifying fluoride-resistant gut bacteria (Liang et al., 2024).

This research aims to isolate and characterize fluoride-resistant bacterial populations from mammalian guts to better understand their diversity, resistance mechanisms, and potential health implications. The findings could offer crucial insights into the ecological and clinical risks of fluoride exposure in humans and aid in addressing related public health challenges.

OBJECTIVES OF THE PRESENT STUDY

i. Preparation of animal models keeping vehicular control

ii. Isolation and purification of fluoride-resistant bacterial strains

iii. Biochemical and molecular characterization of fluoride-resistant bacterial strains

iv. Determination of MAR index using antibiotic-susceptibility test

MATERIALS AND METHODOLOGY

1) Preparation of animal models keeping vehicular control:

❖ **Selection & Design of the Animal Model:**

Rodent models, especially rats, are widely used in research to understand how toxic substances like fluoride affect the gut microbiome. One reason for using rodents is their genetic and gut microbial similarity to humans, which makes them useful for studying how harmful chemicals might impact human health (McDermott et al., 2023; Wang et al., 2023). Studies have shown that the types of bacteria found in the guts of rodents are quite similar to those in humans, which makes the results of such research more reliable and relevant. These models facilitate controlled laboratory experiments, allowing precise regulation of variables such as diet, living conditions, and exposure to contaminants to study their impact on the gut microbiome (Weber et al., 2011).

Standardized rodent strains, like adult Wistar rats are commonly used in scientific research due to their genetic uniformity, which helps maintain consistency in experimental results. Their use reduces biological variability, making it easier to evaluate the specific effects of substances like fluoride on the gut microbiota (Lewi et al., 1982; Weber et al., 2011; Patel et al., 2024). These rats are particularly suited for laboratory studies because they are easy to handle, require minimal care, and have a suitable body size for long-term observation. Such qualities make them an excellent model for investigating how prolonged fluoride exposure influences gut health (de Kort et al., 2020).

❖ **Experimental Design:**

Adult Wistar rats (*Rattus norvegicus*) were sourced from a reputable breeder and approved by the Animal Ethical Committee of Presidency University, Kolkata. Initially, 12 Wistar rats, both males and females, each averaging 120 grams in weight, were housed in polycarbonate cages within the Quarantine Room of the University Animal House Facility, Department of Life Sciences, Presidency University, Kolkata.

Animal Ethical Clearance Number: PU/IAEC/SM/52

The rats were kept in a controlled laboratory environment with a temperature precisely maintained at 22 degrees Celsius, with a possible variation of ± 2 degrees. Humidity levels were maintained at 45%, with an allowable variation of ± 5%. The rats experienced a 12-hour light/dark cycle and had unrestricted access to drinking water, along with a standard food-pellet diet (refer to Table for diet composition).

After being placed under observation for a two-week quarantine period to monitor for any signs of illness or stress, only the Wistar rats that met all the established health and physiological criteria were selected for further use in the study. These healthy animals were then carefully relocated to the designated Rat Room within the Animal House Facility at Presidency University. This selection process was crucial to ensure that only fit and stable individuals participated in the experimental procedures. By implementing strict health screening and controlled housing conditions, the researchers ensured the integrity and accuracy of the experimental outcomes while prioritizing animal welfare. Such rigorous pre-experimental care helps reduce variability in results and aligns with ethical standards for animal research.

Sl. No.	**Ingredients**	**Total amount (in gm)**	**Carbohydrate**	**Protein**	**Lipid**	**Mineral and vitamin**
1	**Wheat flour**	1500	1155	165	30	150
2	**Roasted Bengal gram flour**	1000	650	260	60	30
3	**Sucrose**	750	748.5	0.75	0.75	0
4	**Casein**	200	4	190	0	6
5	**Refined groundnut oil**	150	0	0	150	0
6	**Salt mixture**	50	0	0	0	50
	Total (in gm)	**3650**	**2557.5**	**615.75**	**240.75**	**236**
	Present %		**70.068**	**16.87**	**6.596**	**6.466**
	Expected %		**71**	**18**	**7**	**4**

Table 2: Composition of Rat Diet

❖ **Treatment Procedure:**

In this experiment, a total of 12 rats were selected and evenly divided into two experimental groups. Group I, which served as the control, consisted of 3 male and 3 female rats. Group II, the treatment group, was similarly composed of 3 males and 3 females, but these rats were subjected to oral administration of sodium fluoride (NaF). Throughout the study duration, both groups were closely observed to monitor any physiological or behavioral changes.

Sodium fluoride was administered to Group II at a dosage of 10 mg per kg of body weight per day, continuing for 90 days to simulate sub-chronic exposure (Kumar Sharma et al., 2021; Mehrzadi et al., 2021). This dosage was selected based on its representation of approximately 20% of the lethal dose (LD50) for NaF, ensuring safety while still enabling observable biological effects (Bashir et al., 2006; AAT Bioquest, Inc., 2023).

To accurately simulate human fluoride exposure in a rodent model, the dosage was calculated using established interspecies dose conversion methods. These take into account differences in metabolic activity and body surface area between humans and rats. Fluoride contamination levels recorded in West Bengal, India, especially those reported by the Central Ground Water Board (2009), were used as a baseline reference to determine a realistic environmental exposure level.

❖ **Tissue Collection:**

At the conclusion of the exposure period, the animals were ethically euthanized using ketamine at a dosage of 10 mg/kg body weight, in accordance with established protocols (Bhatia et al., 2022). Following euthanasia, biological samples including gut tissue, blood, and other organs were carefully collected. Specifically, 2 ml of whole blood was extracted from each rat, and representative sections of the gastrointestinal tract were isolated for further study. All subsequent experiments involving mammalian gut bacterial isolates were conducted within certified Biosafety Cabinets, ensuring adherence to rigorous biosafety and procedural standards.

2) Isolation and Purification of Antibiotic-Resistant Strains:

Following aseptic procedures to avoid contamination, different sections of the gastrointestinal sample were collected and preserved in 4 Eppendorf tubes filled with 1X PBS buffer. The gut samples were manually homogenized to ensure uniform bacterial suspension. Afterward, 250 µl of each suspension (duodenum, jejunum, ileum, and caecum) were combined to obtain a total volume of 1 ml suspension.

The homogenized gut contents collected from each experimental group were carefully diluted in a stepwise manner using sterile phosphate-buffered saline (PBS) to reduce bacterial concentration for easier enumeration. Equal volumes from each dilution were then aseptically spread onto nutrient agar (NA) plates under sterile conditions. These plates were incubated at an appropriate temperature to allow visible bacterial colonies to develop. After incubation, plates with clearly countable and well-separated colonies were selected for further microbial analysis, ensuring accurate isolation and identification of the gut bacteria.

Apart from quantifying total culturable gut bacteria, bacterial populations displaying resistance to fluoride (F^-) were isolated from various experimental groups of model organisms. This isolation was accomplished by subjecting the bacterial populations to selective pressures on the Mueller-Hinton Agar (MHA) medium. These pressures were tailored to assess arsenic resistance. To evaluate fluoride resistance, bacteria were cultured on MHA medium supplemented with 25 mM NaF, in accordance with the method described by Banerjee et al. (2011). In all experimental groups, both the control and fluoride group, bacterial strains resistant to fluoride were isolated. This selective isolation approach facilitated the characterization of bacterial populations specifically adapted to fluoride exposure.

Following the initial isolation process, colonies displaying distinct characteristics such as size, shape, colour, and texture were identified for further isolation. To obtain pure bacterial cultures from mixed populations, the streak plate technique was utilized. After the incubation period, pure, individual bacterial colonies were obtained for further analysis. Bacterial isolates were stored in stab culture at room temperature and in glycerol stock at -20 degree Celsius.

3) Characterization of Antibiotic Resistant Strains:

❖ Biochemical Characterization:

Biochemical testing is fundamental in microbiology for the identification and classification of bacterial isolates. These assays assess the metabolic processes of bacteria, particularly through the detection of specific enzymes they produce. Observable changes such as colour shifts, gas formation, or other reactions indicate the presence or absence of these enzymes, helping differentiate bacterial species. Commonly used tests include the catalase test, oxidase test, indole production, and various carbohydrate fermentation assays. The results form a biochemical profile unique to each organism, which can then be matched against reference databases for accurate species identification. These tests are crucial in numerous areas, including clinical microbiology, environmental studies, microbial taxonomy, and industrial applications.

Biochemical tests performed are as follows:

1. **Catalase Test:**

- **Principle:** The catalase test is used to detect the presence of the catalase enzyme, which facilitates the decomposition of hydrogen peroxide into water and oxygen.
- **Reagents/Media Composition**: Hydrogen peroxide solution (3%)
- **Methodology:** Isolated individual colonies from a nutrient agar plate were transferred onto a microscope slide. A 1% solution of hydrogen peroxide (H_2O_2) was then carefully dispensed onto the colonies using a glass capillary tube. The observation of effervescence (bubbling) indicated the activity of the catalase enzyme.
- **Positive result:** Immediate bubble formation.
- **Negative result:** No Bubble.

2. **Oxidase Test:**

- **Principle:** This test detects the presence of cytochrome c oxidase, an enzyme involved in the bacterial electron transport chain.
- **Reagents/Media Composition:** Oxidase reagent disks (DD 018; Himedia Laboratories)
- **Methodology**: Individual bacterial colonies were selected from a nutrient agar plate maintained at pH 7.0. Using a sterile toothpick, the colonies were gently rubbed onto the surface of the oxidase disk, which acts as an electron donor to cytochrome oxidase.
- **Positive result:** Dark purple/blue colour within 10-30 seconds.
- **Negative result:** No visible color transformation or a delayed color shift observed beyond 30 seconds.

3. **Indole Test:**

- **Principle:** This test is used to determine whether a bacterial strain can break down the amino acid tryptophan into indole with the help of the enzyme tryptophanase.
- **Reagents / Media Composition:**
  Tryptone broth: - Tryptone: 10gm/L, Sodium chloride (NaCl): 5gm/L
  Kovac's reagent: - Amyl or isoamyl alcohol: 150.0 ml, p-dimethyl aminobenzaldehyde (DMAB):10.0 gm, Conc.HCl: 50.0 ml
- **Methodology:** Individual bacterial colonies grown on nutrient agar were aseptically transferred into sterile test tubes containing 4 ml of tryptone broth. The inoculated tubes were then incubated at 37°C for a period of 24 to 28 hours. Following incubation, 0.5 ml of Kovac's reagent was gently added to each culture to assess indole production.
- **Positive result:** Formation of a cherry-red ring in the reagent layer on top of the medium within seconds of adding the reagent.
- **Negative result:** No colour changes even after the addition of Kovac's reagent.

4. **Gelatin Hydrolysis Test:**

- **Principle:** Determines the ability of bacteria to hydrolyze gelatin using the enzyme gelatinase
- **Reagents/Media Composition:** Enzymatic digest of gelatine/peptone: 5 gm, Beef extract:3 gm, Gelatine 120 gm, per 1000 mL, pH 6.8.
- **Methodology:** Using an inoculating loop, a small amount of the bacterial culture was picked and stabbed into a gelatin medium tube. Inoculated tubes along with an un-inoculated medium were incubated at 35°C for 48 hours. Liquefaction of the medium was checked after placing the tube in the refrigerator for 30 minutes
- **Positive result:** Liquefaction of gelatin (liquid after refrigeration)
- **Negative result:** Solid gelatin (no liquefaction)

5. **Methyl-Red (MR) Test:**

- **Principle:** Measures production of stable acid end products from glucose fermentation.
- **Reagents / Media Composition:**
  MR-VP broth: 7g/L peptone,5g/L glucose, 5g/L dipotassium phosphate; pH 6.9
  Methyl red indicator: 0.1% methyl red in 300 ml ethanol (95%) & add 200 ml deionized water.
- **Methodology:** A light inoculum from an isolated colony was transferred in the 5 ml MR-VP broth tube. After 48 hours of incubation, 2.5 ml of bacterial culture was transferred to a new tube. 5 drops of methyl red indicator were added and the observations were noted to make the interpretation.
- **Positive result:** Red colour (pH < 4.4). Examples: *E. coli, Yersinia sp.*, etc.
- **Negative result:** Yellow colour (pH > 6.0) Example: *Enterobacter aerogenes*

6. **Voges-Proskauer (VP) Test:**

- **Principle:** Detects acetoin production from glucose fermentation
- **Reagents / Media Composition:**
  MR-VP broth: 7g/L peptone, 5g/L glucose, 5g/L dipotassium phosphate;
  VP reagents: Barritt's reagent A: 5% (wt/vol) alpha naphthol in absolute ethanol. Barritt's reagent B: 40% (wt/vol) KOH in deionized water.
- **Methodology:** The remaining 2.5 ml of culture grown in MR-VP broth was used for the VP test. 0.6 ml of Barritt's reagent A & 0.2 ml of Barritt's reagent B were added to the tubes. After carefully shaking the tubes for 30 seconds to 1 minute, tubes were kept to stand for at least 30 minutes before interpretation. Exposing the medium to atmospheric oxygen is necessary for the oxidation of acetoin to obtain a color reaction.
- **Positive result:** Red colour within 30 minutes. Example: *Enterobacter aerogenes, Proteus mirabilis*
- **Negative result:** No color change or copper color. Example: *E. Coli, Shigella sp.*

7. **Triple Sugar Iron (TSI) Test:**

- **Principle:** Differentiates bacteria based on glucose, lactose & sucrose fermentation and sulfur reduction. Gas production (CO_2 and O_2) is detected by splitting of the agar. H_2S production is detected by black precipitation at the bottom.
- **Reagents/Media Composition:**
  TSI agar slant: 1gm/L glucose, 10 gm/L lactose, 10 gm/L sucrose, 3 gm/L beef extract, 3 gm/L yeast extract, 20 gm/L peptone, 0.2 gm/L ferrous sulfate, 0.3 gm/L sodium thiosulfate, 5 gm/L NaCl, phenol red as pH indicator, 0.024 gm/L, 15 gm/L agar.
- **Methodology:** Using a straight inoculating needle an isolated colony was picked up. Then with the needle, the butt was first stabbed to the bottom. After withdrawing the needle, the slant was streaked using the same needle. A loosely fitting closure was capped to permit access to air. Results were interpreted after incubation at 37°C for 18 to 24 h.
- **Positive result:** Yellow butt/red slant (Glucose); Yellow butt/Yellow slant (Glucose, Lactose, or Sucrose); Black precipitate H_2S production; Crack in the media (gas produced)
- **Negative result:** Red butt/red slant (no fermentation), no black precipitation or gas.

8. **Citrate Utilization Test:**

- **Principle:** This test determines the ability of an organism to use citrate as its sole carbon source and ammonium ions as its sole nitrogen source.
- **Reagents / Media Composition:**
  Simmon's Citrate Agar: Sodium Chloride 5.0 gm/L, Sodium Citrate (dehydrated)- 2.0 gm/L, Ammonium Dihydrogen Phosphate 1.0 gm/L, Dipotassium Phosphate 1.0 gm/L, Magnesium Sulfate (hepta hydrate) 0.2 gm/L, Bromothymol Blue 0.08 gm/L, Agar 15.0 gm/L
- **Methodology:** A single isolated colony was picked from a fresh culture and lightly streaked on the surface of the slant. After loosely closing the tube, the medium was incubated at 37°C for up to 7 days. Any change in the color of the medium was monitored every day till day 7. Some organisms are capable of growth on citrate and do not produce a color change. Growth was considered a positive citrate utilization test, even in the absence of a color change.
- **Positive result:** Growth on the medium and/or a blue color change indicates citrate utilization. Examples: *Salmonella, Citrobacter, Klebsiella, Enterobacter, Serratia,* etc.
- **Negative result:** No growth and the medium remaining green indicates citrate non-utilization. Examples: *E coli, Shigella, Morganella, Yersinia* etc.

▪ Gram Staining:

Principle:

Gram staining is a differential staining technique used to distinguish bacteria as either Gram-positive or Gram-negative based on differences in their cell wall structure. In Gram-positive bacteria, the thick peptidoglycan layer retains the crystal violet-iodine complex, making them

appear purple. In contrast, Gram-negative bacteria have a thinner peptidoglycan layer that does not hold the dye after the decolorization step, allowing them to take up the counterstain (such as safranin or fuchsin) and appear pink or red.

Reagents / Media Composition:

- Primary stain: Crystal violet (1% aqueous solution)
- Mordant: Iodine solution (1% aqueous solution)
- Decolorizing agent: 95% ethanol or acetone-alcohol mixture
- Counterstain: Safranin (0.25% aqueous solution)

Procedure:

i. A thin bacterial smear was prepared on a clean glass slide and heat-fixed to ensure adhesion.
ii. The slide was stained with crystal violet for approximately 20 seconds, and then gently rinsed with water.
iii. Next, Gram's iodine was applied for 1 minute to form a dye-mordant complex, followed by a water rinse.
iv. The smear was decolorized using ethanol or an acetone-alcohol solution for 10 to 30 seconds and rinsed again.
v. Finally, the slide was counterstained with safranin for 1 minute, rinsed with water, and air-dried.

Interpretation:

- **Gram-positive Bacteria:** Retain the primary stain (crystal violet) and appear purple or blue under a microscope.
  Examples: *Bacillus, Enterococcus, Lactobacillus, Listeria, Nocardia, Staphylococcus, Streptococcus, Streptomyces,* etc.
- **Gram-negative Bacteria:** Lose the crystal violet stain and take up the counterstain (safranin) and appear pink or red**.**
  Examples: *Escherichia coli, Salmonella, Shigella, Pseudomonas, Helicobacter,* etc.

❖ Molecular Characterization:

- **Bacterial Genomic DNA Isolation**:
  To begin with genomic analysis, different bacterial isolates were inoculated into test tubes containing 5ml Luria Broth (LB) and was allowed to incubate at 37°C for 24 hours. Total bacterial Genomic DNA was extracted from those bacterial liquid cultures with the help of the bacterial Genomic DNA Purification Kit (HiPurA® Bacterial Genomic DNA Purification Kit). The kit employs a silica-based membrane technology within a spin column, streamlining the processes of binding, washing, and elution to allow for the simultaneous processing of multiple samples. It consists of three key steps:

i. Binding of DNA to the membrane surface,

ii. Removal of residual contaminants, and
iii. Elution of purified genomic DNA from the membrane.

Following extraction, the concentration and purity of the extracted DNA were evaluated using a spectrophotometer, while its integrity and quantity were determined by running it on a 0.8% agarose gel through electrophoresis.

- **PCR Amplification of 16S rRNA Genes:**

The isolated genomic DNA was subjected to PCR amplification of the 16S rRNA gene. Each 50µl reaction volume was prepared by adding-

i. 2 µl of forward primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′),
ii. 2 µl of reverse primer 1492R (5′-GGTTACCTTGTTACGACT T -3′),
iii. 1 µl of sample DNA (20 ng),
iv. 20µl of molecular grade water, and
v. 25µl of PCR master mix.

The master mix is composed of

- DNA polymerase: This enzyme synthesizes new strands of DNA.
- dNTPs (deoxynucleotide triphosphates): Building blocks of new DNA strands.
- $MgCl_2$: A cofactor that is required for the activity of the DNA polymerase.
- Buffer: This maintains the optimal pH and salt conditions for the PCR.

The PCR setup was -

- Initial denaturation at 94°C for 4 minutes.
- Denaturation at 94°C for 1 minute
- Annealing at 55°C for 1 minute } 30 cycles
- Extension at 72°C for 2 minutes
- Final Extension at 72°C for 10 minutes
- Soak/Hold at 4°C
- Lid temperature set at 104°C.

The products were visualized on a 1 % agarose gel. PCR products were gel purified by Himedia gel purification kit and outsourced for sequencing by using the paid sequencing facility from Nalgen Biotech.

4) Determination of Antibiotic-Susceptibility test using Antibiogram Analysis:

Determination of Antibiogram and Multiple Antibiotic Resistance (MAR) index of the strains:

Antibiotic susceptibility test: Antibiotic susceptibility testing was performed by the disc diffusion method with commercially available disks from HiMedia®, India. The selected antibiotics were:

Gentamicin (10 µg), Amikacin (30 µg), Netillin (30 µg), Vancomycin (30 µg), Oxacillin (1 µg), Ampicillin (10 µg) , Cefazolin (30 µg), Cephalothin (30 µg), Cefoxitin (30 µg) ,

Cefuroxime(30 µg), Ceftazidime (30 µg), Cefixime (5 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Cefoperazone (75 µg), Cefepime (30 µg), Aztreonam (30 µg), Tetracycline (30 µg), Norfloxacin (10 µg), Ciprofloxacin (5 µg), Levofloxacin (5 µg) , Erythromycin (15 µg), Cotrimoxazole (25 µg), Polymyxin B (300 unit), Colistin (10 µg), Clindamycin (2 µg).

Sl. No.	**Antibiotic Name**	**Generation**	**Class/ Category**	**Mode of action**
1	Gentamicin (GEN)	2	Aminoglycosides	inhibit bacterial protein synthesis by binding to the 30S ribosomal subunit
2	Amikacin (AK)	3		
3	Netillin (NET)	3		
4	Vancomycin (VA)	1	Glycopeptides	inhibit cell wall synthesis by binding to D-Ala-D-Ala terminus
5	Oxacillin (OX)	2	Penicillin	inhibit bacterial cell wall synthesis by binding to PBPs
6	Ampicillin (AMP)	3		
7	Cefazolin (CZ)	1	Cephalosporin	inhibit bacterial cell wall synthesis by binding to penicillin-binding proteins (PBPs)
8	Cephalothin (CEP)	1		
9	Cefoxitin (CX)	2		
10	Cefuroxime (CXM)	2		
11	Ceftazidime (CAZ)	3		
12	Cefixime (CFM)	3		
13	Cefotaxime (CTX)	3		
14	Ceftriaxone (CTR)	3		
15	Cefoperazone (CPZ)	3		
16	Cefepime (CPM)	4		
Sl. No.	Antibiotic Name	Generation	Class/Category	Mode of Action
17	Aztreonam (AT)	1	Monobactam	inhibits cell wall synthesis by interfering with peptidoglycan synthesis
18	Tetracycline (TE)	1	Tetracyclin	inhibit bacterial protein synthesis by binding to the 30S ribosomal subunit
19	Norfloxacin (NX)	1		

20	Ciprofloxacin (CIP)	2	Quinolones/ Fluoroquinolones	inhibit DNA synthesis by targeting bacterial DNA gyrase and topoisomerase IV
21	Levofloxacin (LE)	3		
22	Erythromycin (E)	1	Macrolides	inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit
23	Cotrimoxazole (COT)	__	Sulphonamides	inhibit the bacterial folic acid synthesis
24	Polymyxin B (PB)	1	Polymyxins	disrupt bacterial cell membranes by interacting with phospholipids
25	Colistin (CL)	1		
26	Clindamycin (CD)	2	Lincosamides	inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit
27	Chloramphenicol (C)	__	Amphenicols	inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit

Table 3: Summary of the selected 27 antibiotics

- **Experiments Performed:**

  - Along with total culturable gut bacteria, Fluoride-tolerant bacterial populations were isolated from different groups of model animals by applying selection pressure on Mueller-Hinton Agar (MHA) media. Different F-tolerant bacterial strains were purified from the above-mentioned culture plate with the help of the Streak Plate method (Duranti et al., 2017).
  - Liquid culture suspensions of those purified well-isolated colonies were obtained after inoculation of a single isolated colony into test tubes containing 5ml Mueller-Hinton Broth (MHB) and incubation at 37°C in an orbital shaker at 120 rpm for 12 hours. After 12 hours of incubation, the cell density of each tube was measured by a spectrophotometer at 660 nm. After calculating the bacterial cell density, 1 McFarland amount of cell suspension was applied on Mueller-Hinton Agar (MHA) plates and 27 different Gibco^{TM} antibiotic discs were placed on it. The zone diameter for each antibiotic disc was measured after 24 hours of incubation at 37°C.
  - Isolates were considered susceptible, moderately susceptible, or resistant to a particular antibiotic disc based on the diameters of the inhibitory zones that matched the criteria of the manufacturer's interpretive table, which followed the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) (Maden et al., 2011; Shakya et al., 2012).
  - The MAR Index of those enumerated antibiotic-resistant bacteria was calculated from the acquired data with the help of the interpretive table that is recommended by NCCLS (Thakurta et al., 2007).

RESULTS & DISCUSSIONS

- **Isolation and Purification of Fluoride-Tolerant Bacterial Strains:**

  From a total of 12 Wistar rat gut samples, we have successfully isolated and purified different bacterial strains based on colony morphology, basic microscopic observation, and some biochemical tests. From control and Fluoride-treated groups, we have isolated different bacterial isolates respectively.

Strain Code	Catalase Test	Oxidase Test	Indole Test	Gelatin Hydrolysis Test	Citrate Utilization Test	MR-VP Test		TSI Test		Gram Staining & Colony Morphology
						MR Test	VP Test	Gas	H2S	
AF1F-a	Positive	Negative	Positive	Positive	Positive	Positive	Negative	(+)	(+)	Negative rods
AF2F-b	Positive	Negative	Negative	Positive	Positive	Positive	Negative	(-)	(-)	Positive cocci
AM3F-a	Positive	Negative	Negative	Negative	Positive	Positive	Negative	(-)	(-)	Positive cocci
AF3F-a	Positive	Negative	Negative	Negative	Positive	Positive	Negative	(-)	(-)	Positive cocci
CM1F-c	Positive	Negative	Negative	Negative	Positive	Positive	Negative	(-)	(-)	Positive cocci
CM3F-a	Positive	Negative	Negative	Positive	Positive	Positive	Negative	(-)	(-)	Positive cocci
CF3F-a	Positive	Negative	Negative	Positive	Positive	Positive	Positive	(-)	(-)	Positive cocci
CF1F-b	Positive	Negative	Negative	Negative	Positive	Negative	Positive	(-)	(-)	Positive cocci

Table 4: Results of Biochemical Tests of Isolated Bacterial Strains [Positive (+); Negative (-)]

- **Differential abundance of total viable and F-tolerant bacterial strains:**

  The following table (Table 5) shows the differential abundance of total viable and F-tolerant bacterial strains in gut samples of different model animals of each group in terms of Colony Forming Unit (CFU) per ml. Here, due to the addition of selection pressure, colony count is decreasing in gut samples of each individual model animal.

Animal Group		Colony count without HM selection pressure (CFU/ml)	Colony count in Fluoride selection pressure (CFU/ml)
Control	Male 1	161000	122200
	Male 2	723000000	360000000
	Male 3	500000	140000
Total of Control Males:		723661000	360262200
Control	Female 1	34000	17900
	Female 2	70700000	52300000
	Female 3	7400	6300

Total of Control Females:		70741400	52324200
F-Treated	**Male 1**	16000	14000
	Male 2	252000000	216000000
	Male 3	433000	209000
Total of Treated Males:		252449000	216223000
F-Treated	**Female 1**	17000	5400
	Female 2	439000000	162000000
	Female 3	2720	14100
Total of Treated Females:		439019720	162019500

Table 5: Differential abundance of total viable and F-tolerant bacterial strains in gut samples of different model animals of each group

If we only consider the 2 main groups of model animals irrespective of their male and female individual, the following data will be obtained (Table 6). In both groups, there was a reduction in number of CFU in the presence of Fluoride selection pressure.

Animal Group	**Total Colony Count in MHA without F (CFU/ml)**	**Total Colony Count in MHA with 10Mm F (CFU/ml)**
Control Group	794402400	412586400
F-Treated Group	691468720	378242500

Table 6: Differential abundance of total viable and F-tolerant bacterial strains in the Control and F-treated group

Though the F-treated group shows a drastic reduction in total colony count in comparison to the control group, the reduction rate in the number of F-resistant bacterial isolates is quite low (Figure 2).

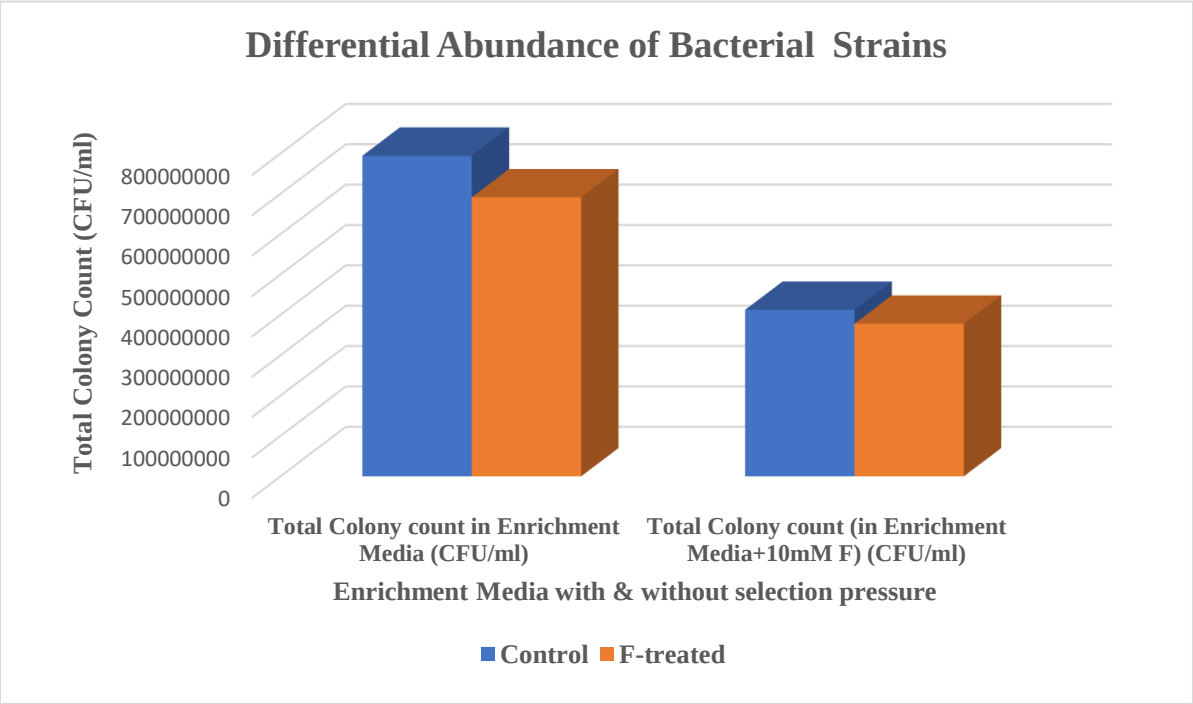


Figure 2: Differential abundance of total colony count in Enrichment media and F-selection pressure of Control and F-treated group

Fluoride treatment appears to reduce the total gut bacterial population under normal conditions but may promote the survival of fluoride-tolerant strains when exposed to fluoride selection pressure. This adaptation could be the result of prolonged fluoride exposure selecting for more resilient bacterial populations.

- ## Results of Antibiotic Susceptibility Test:

  Determination of antibiogram was performed by antibiotic susceptibility test. After preparing 5ml Mueller-Hinton Broth liquid culture suspension of those purified well-isolated colonies, the cell density of each tube was measured by a spectrophotometer at 660 nm. After calculating the bacterial cell density, 1 McFarland amount of cell suspension was applied to Mueller-Hinton Agar (MHA) plates and the different Gibco^{TM} antibiotic discs were placed on them. The zone diameter for each antibiotic disc was measured after 24 hours of incubation at 37°C. Isolates were considered sensitive, moderately sensitive, or resistant to a particular antibiotic disc based on the diameters of the inhibitory zones that matched the criteria of the manufacturer's interpretive table, which followed the recommendations of the NCCLS (Maden et al., 2011; Shakya et al., 2012).

Group	Strain No.	Number of Antibiotics to which a particular strain is showing		
		Sensitive (S)	Intermediate (I)	Resistant (R)
Control	1	13	3	11
	2	13	6	8
	3	13	2	12
	4	24	1	2
	5	23	1	3
	6	15	1	11
	7	16	2	9
	8	17	5	5
	9	22	3	2
	10	21	4	2
	11	20	3	4
	12	19	5	3
	13	25	0	2
	14	22	1	4
	15	23	1	3
	16	20	4	3
F-treated	1	8	1	18
	2	10	2	15
	3	9	5	13
	4	21	2	4
	5	14	2	11
	6	13	2	12
	7	23	2	2
	8	20	4	3
	9	22	1	4
	10	21	3	3
	11	22	1	4
	12	15	7	5
	13	18	7	2
	14	13	8	6
	15	17	5	5
	16	18	4	5
	17	21	2	4
	18	19	2	6
	19	19	5	3
	20	19	3	5
	21	21	3	3
	22	19	3	5

Table 7: Results of Antibiotic Susceptibility Test of each bacterial isolates of Control and F-treated groups

Animal Group	Average Number of Antibiotics					
	Sensitive(S)	SD	Intermediate(I)	SD	Resistant(R)	SD
Control	19.125	3.982	2.625	1.727	5.25	3.52
F-Treated	17.36	4.333	3.363	1.989	6.27	4.38

Table 8: Summary of the results of the Antibiotic Susceptibility Test of Control and F-treated groups

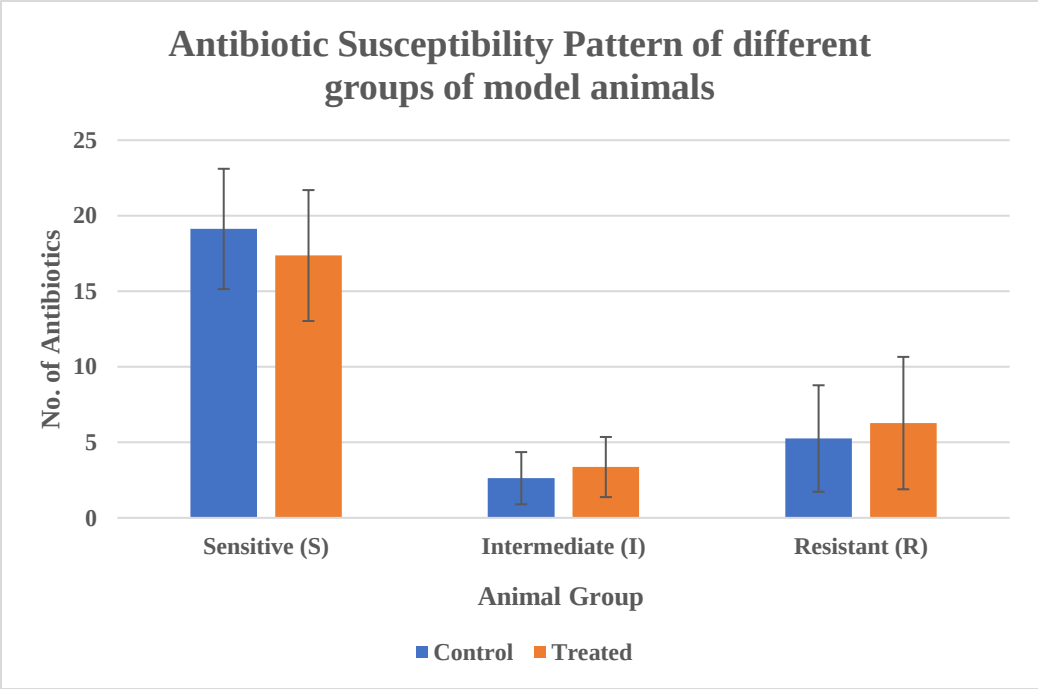


Figure 3: Graphical representation of the results of the Antibiotic Susceptibility Test of Control and F-treated groups

In Figure 3, Fluoride-treated animals show slightly more resistance to antibiotics (lower sensitivity) than untreated ones. However, for strong resistance and intermediate response, both groups behave similarly. This might indicate that fluoride exposure could contribute to a small increase in antibiotic resistance.

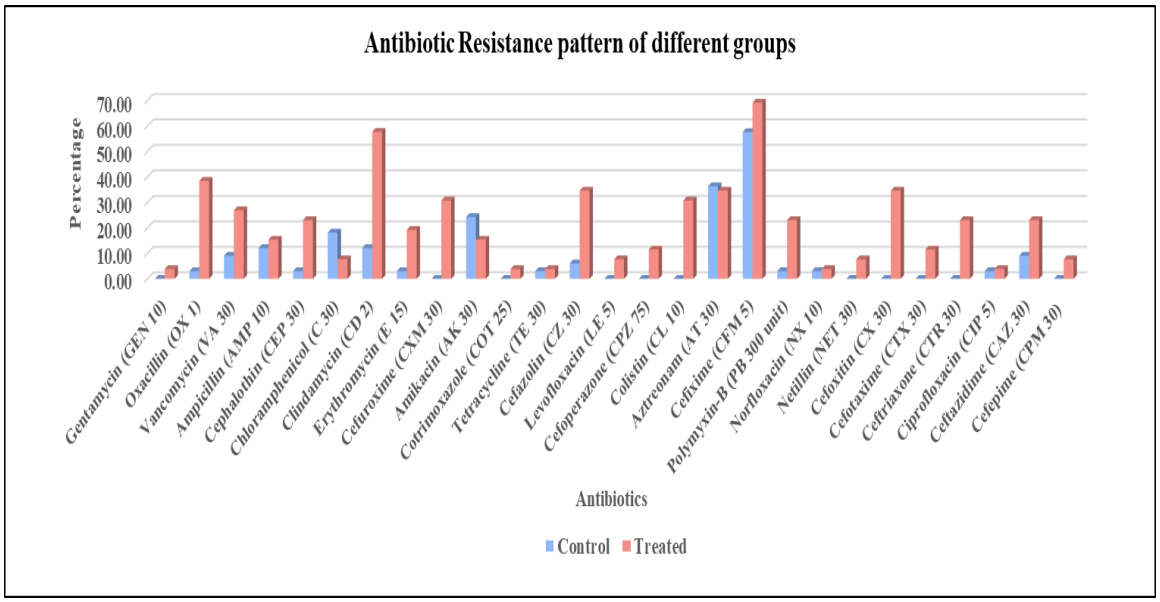


Figure 4: Comparative study of Antibiotic Resistance pattern in between Control and F-treated groups

Bacterial isolates from the treated group were resistant to all the 27 antibiotics tested, but the control group isolates were resistant to only 16 (Figure 6), indicating a broader resistance spectrum. The MAR Index of those enumerated antibiotic-resistant bacteria was calculated from the acquired data with the help of the interpretive table that is recommended by NCCLS (Thakurta et al., 2007).

Group	MAR Index
Control	0.097
F-Treated	0.124

Table 9: Calculated MAR Index of Control and F-Treated groups

The observed calculations in Table 9 provide the insight that the MAR Index values indicate that the F-treated group has a higher MAR index than the control group. This suggests that fluoride treatment may contribute to increased antibiotic resistance in the bacterial population. A higher MAR index reflects a greater exposure or adaptation to antibiotics, possibly due to co-selection pressure, where fluoride exposure may select for strains that also carry antibiotic resistance traits.

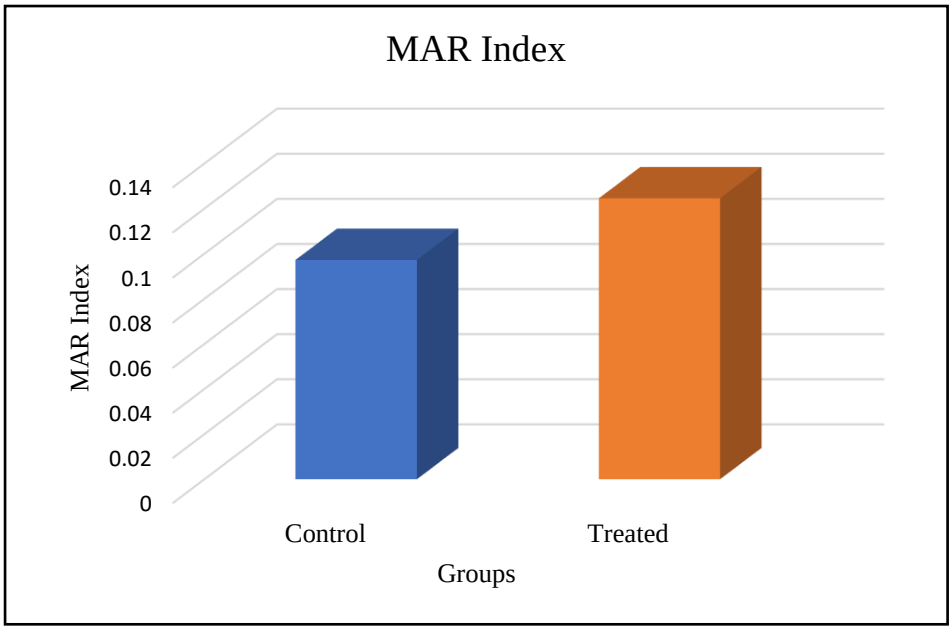


Fig 5: Graphical representation of calculated MAR Index of Control and As-treated groups

SUPPLEMENTARY DATA

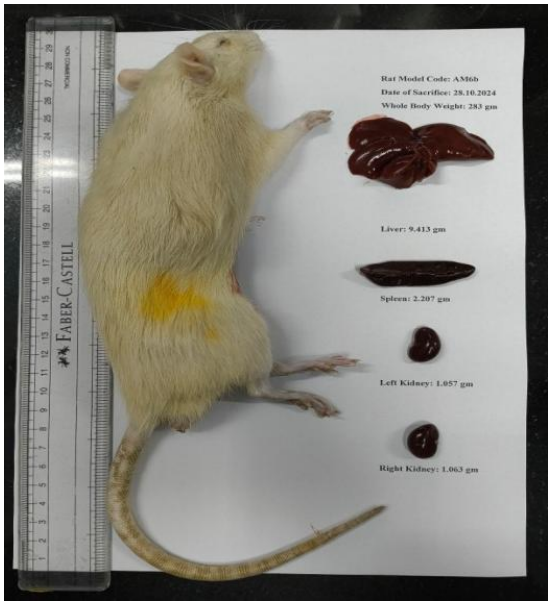


Fig 6: Control Wistar Rat model sacrifice showing its height and separated organs for calculating Mean Organ Index

Fig 7: Fluoride-Treated Wistar Rat model sacrifice showing its height and separated organs for calculating Mean Organ Index

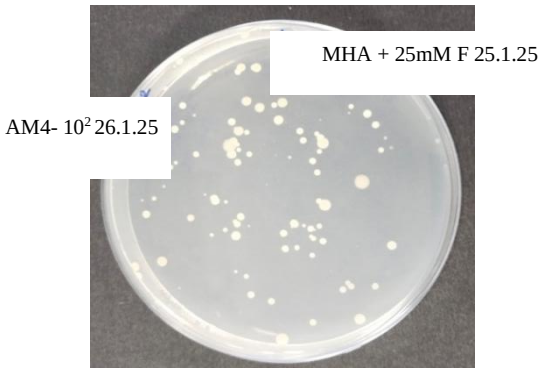


Fig 8: Isolation of fluoride-tolerant bacterial strains

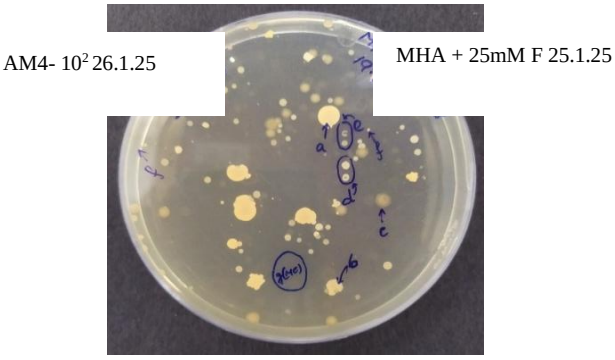


Fig 9: Characterization of fluoride-tolerant bacterial strains in terms of colony morphology

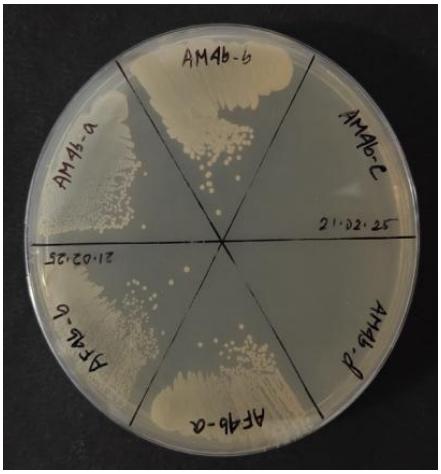
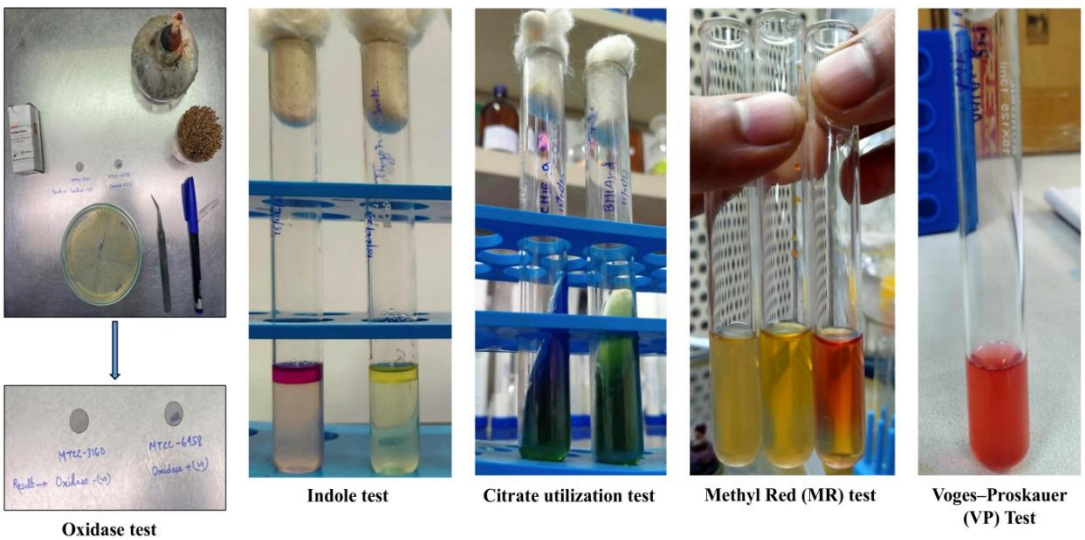


Fig 10: Purification of fluoride-tolerant bacterial strains by Streak plate method



Oxidase test | Indole test | Citrate utilization test | Methyl Red (MR) test | Voges–Proskauer (VP) Test

Fig 11: Results of Different Biochemical Tests conducted for the Isolated Bacterial Strains

Fig 12: Results of Conducted Gram Staining for the Isolated Bacterial Strains

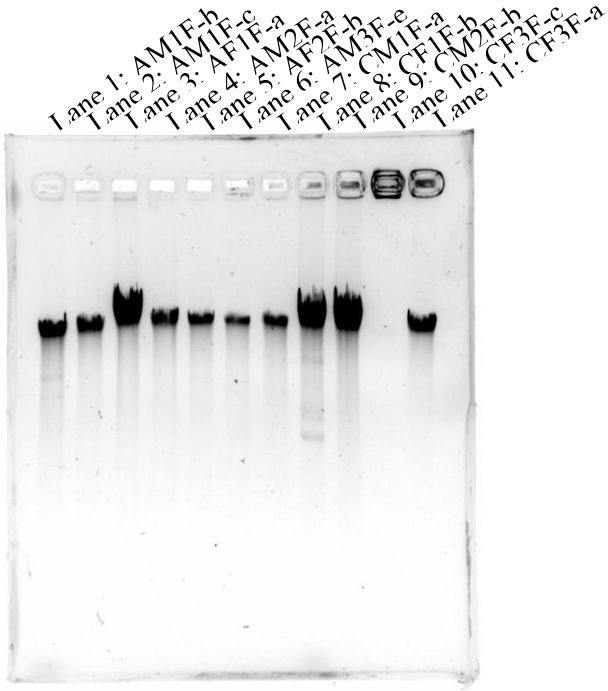


Fig 13: Molecular Identification by Isolation of Genomic DNA from different Isolated Bacterial Strains

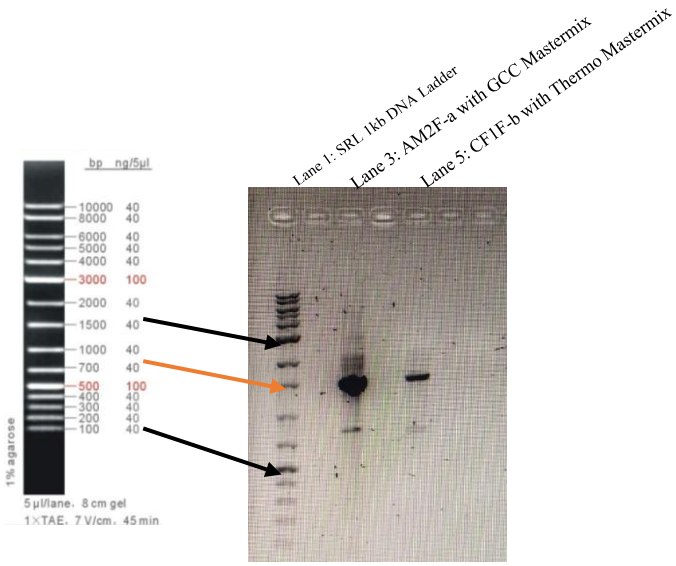


Figure 14: Result of Polymerase Chain Reaction (PCR) of Isolated Bacterial Genomic DNA

Figure 15: Selected Choice of Antibiotic combinations

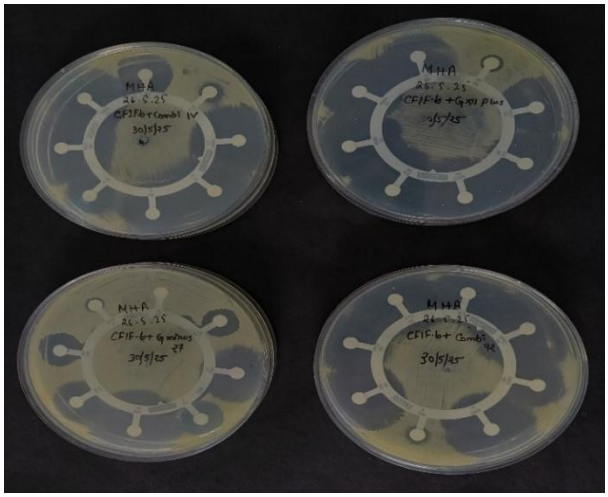


Figure 16: Determination of sensitivity of the arsenic-resistant bacteria for different antibiotics by the standard disc-agar diffusion method on Mueller-Hinton Agar (MHA) plates

CONCLUSION

This investigation focused on isolating and characterizing fluoride-resistant bacterial strains from the mammalian gut, using Wistar rats as the experimental model. Over the course of the study, the animals were subjected to sub-chronic sodium fluoride exposure, which not only altered the gut microbial composition but also encouraged the proliferation of strains resistant to both fluoride and multiple antibiotics. These findings underscore the growing concerns surrounding environmental pollution and the rise of antimicrobial resistance, both of which present substantial risks to public health.

The gut microbiota plays a fundamental role in supporting host health by aiding in digestion, modulating the immune response, and synthesizing vital metabolites. However, the introduction of environmental toxins such as fluoride can disturb this microbial harmony—a condition referred to as dysbiosis. In this study, fluoride exposure created a selective pressure that favoured the survival of fluoride- and antibiotic-resistant strains in the treated group, in contrast to the control group. This resulted in a notable reduction in the population of beneficial gut microbes, impairing normal gut functionality.

A total of 38 bacterial isolates demonstrating resistance to fluoride were identified from the intestinal tracts of the rats and further analysed for both biochemical and molecular traits. Antibiotic susceptibility testing revealed a stark difference between the two groups: while the control group strains exhibited resistance to 16 of the 27 antibiotics tested, the fluoride-treated group showed resistance to all 27. This discrepancy suggests that fluoride exposure not only disrupts the gut ecosystem but also actively contributes to the emergence of multiple antibiotic resistance (MAR) traits. The MAR index for bacteria in the treated group was threefold higher than that of the control, strongly indicating a connection between fluoride presence and the selection for antibiotic resistance.

The outcomes of this study highlight a twofold threat presented by fluoride contamination. First, it destabilizes the gut microbiota, which can lead to a range of health complications. Second, it accelerates the loss of commensal microbial species while encouraging the growth of resistant strains—those capable of withstanding both fluoride and various antibiotics. These alarming trends call for urgent efforts to monitor environmental pollutants and re-evaluate the widespread use of fluoride, particularly in industrial and agricultural applications.

Future research should aim to identify the specific resistance genes responsible for this dual resistance and investigate the long-term health implications of fluoride-induced microbial imbalances. Further studies may also assess gene expression patterns linked to fluoride and antibiotic resistance and explore possible co-regulation between the two. The insights derived from this research could help inform public policy, regulatory decisions, and health interventions aimed at limiting exposure to environmental toxins and combating the escalation of antibiotic resistance.

APPENDIX

Group	Set No.	Culture Plate Code	Strain Code	Numerical Trinomial		
				Sensitive (S)	Intermediate (I)	Resistant (R)
Control	1	AM1F	AM1F-b	13	3	11
			AM1F-c	13	6	8
		AF1F	AF1F-a	13	2	12
			AF1F-b	24	1	2
			AF1F-c	23	1	3
	2	AM2F	AM2F-a	15	1	11
		AF2F	AF2F-a	16	2	9
			AF2F-b	17	5	5
		AM3F	AM3F-a	22	3	2
			AM3F-e	21	4	2
	3	AF3F	AF3F-a	20	3	4
			AF3F-b	19	5	3
			AF3F-e	25	0	2
			AF3F-f	22	1	4
			AF3F-g	23	1	3
			AF3F-i	20	4	3
Treated	1	CM1F	CM1F-a	8	1	18
			CM1F-c	10	2	15
			CM1F-d	9	5	13
		CF1F	CF1F-b	21	2	4
	2	CM2F	CM2F-a	14	2	11
			CM2F-b	13	2	12
			CM2F-c	23	2	2
			CM2F-d	20	4	3
	3	CM3F	CM3F-a	22	1	4
			CM3F-b	21	3	3
			CM3F-c	22	1	4
			CM3F-d	15	7	5
			CM3F-e	18	7	2
			CM3F-f	13	8	6
			CM3F-i	17	5	5
			CM3F-j	18	4	5
			CM3F-k	21	2	4
			CM3F-l	19	2	6
		CF3F	CF3F-a	19	5	3
			CF3F-b	19	3	5
			CF3F-e	21	3	3
			CF3F-f	19	3	5

Table 10: Result of Antibiogram Susceptibility Test

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